NEW INSIGHTS INTO A COMPLEX HOST-PATHOGEN INTERACTION: MECHANISMS OF *HELICOBACTER PYLORI* ACTIVATION OF NF-κB

BY

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DISSERTATION

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ABSTRACT

Helicobacter pylori infection is the main cause of chronic gastritis, gastric ulcers and gastric cancer. The gram-negative spirochete, which infects more than half of the world's population, is recognized by the International Agency for Research on Cancer as a group 1 carcinogen. The mechanism by which *H. pylori* induces carcinogenesis is believed to be its ability to cause chronic inflammation, creating an environment suitable to tumor initiation and progression.

Many different strains of *H. pylori* exist, but those strains harboring the *cag* pathogenicity island (*cag*PAI) have been found to be more virulent, with more carcinomas associated with infection by these strains. Significantly, higher levels of *H. pylori*-initiated chronic gastritis is characterized by the *cag*PAI-dependent up-regulation of proinflammatory cytokines, which are largely mediated by the transcription factor nuclear factor (NF)-κB. The goal of this work is to better define the bacterial proteins and the cellular signaling molecules involved in *H. pylori*-induced NF-κB activation.

The *H. pylori* virulence factor CagA, encoded by the *cag*PAI, is injected into host cells via a type 4 secretion system, where it interacts with a number of different signaling pathways leading to inflammation and cell scattering. While a number of these interactions have been defined, the role of CagA in NF-κB activation and in immune response remains unclear. In this work, CagA is shown to be crucial in the activation of NF-κB by *H. pylori* infection.

Once inside the cell, CagA utilizes host proteins to induce NF-κB activity. CagA associates with transforming growth factor β-activated kinase 1 (TAK1), a MAP kinase involved in the NF-κB, AP-1 and JNK signaling pathways, and enhances the polyubiquitination and

activation of the kinase. This lysine 63-linked ubiquitination is mediated by E3 ligase tumor necrosis factor receptor-associated factor 6 (TRAF6) and E2 Ubc13. These findings show that polyubiquitination of TAK1 regulates the activation of NF-κB, which in turn is used by *H. pylori* CagA for the *H. pylori*-induced inflammatory response.

ACKNOWLEDGEMENTS

I am grateful for the guidance and opportunities given me by my advisor, Dr. Lin-Feng Chen. Many thanks are also owed to the many lab members with whom I worked and from whom I learned a great deal: Dr. Xiao-dong Yang, Dr. Ying-Hung Nicole Tsang, Dr. Bo Huang, Nicholas Liu, and Dr. Xuewei Wu, among others.

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Most importantly, I am thankful for the ever-present love and support of my family and friends, who have encouraged my every accomplishment.

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LIST OF ABBREVIATIONS

AID	Activation-induced cytidine deaminase
CagA	Cytotoxin associated gene A
DNA	Deoxyribonucleic acid
EMSA	Electromobility shift assay
HA	Hemagglutinin
HDAC	Histone deacetylase
IκBα	Inhibitor of kappa B alpha
IKK1/2	Inhibitor of kappa B kinase 1/2
IL-8	Interleukin-8
MARK2	Microtubule-affinity regulating kinase 2
MEF	Mouse embryonic fibroblasts
NF-κB	Nuclear factor kappa B
RHD	Rel homology domain
RNA	Ribonucleic acid
RT-PCR	Real-time polymerase chain reaction
siRNA	Short interfering ribonucleic acid
STAT3	Signal transducer and activator of transcription 3
T4SS	Type 4 secretion system
TAK1	Transforming growth factor-beta activating kinase 1
TNF-α	Tumor necrosis factor-alpha
TSA	Trichostatin A
TRAF6	Tumor necrosis factor receptor-associated factor 6
VacA	Vacuolating toxin A
WT	Wild type

CHAPTER 1

BACKGROUND

1.1 *Helicobacter pylori* and gastric cancer

Helicobacter pylori is a Gram-negative spirochete which infects more than half of the world's population. Infections are very common in undeveloped countries, likely due to water contamination and less sanitary living conditions. The bacterium colonizes the stomach of its host, where it attaches to the mucosal epithelia. Infection persists often for the lifetime of the host unless treatment is received. *H. pylori* is susceptible to most antibiotics, though resistance is becoming more common and triple or quadruple therapy consisting of two antibiotics, a proton pump inhibitor and bismuth is now used to eradicate the bacteria.

There are many different strains of *H. pylori*, and a variety of pathogenicity factors produced by the different strains, creating a great variability in the bacteria's ability to cause disease. Two of the most well studied pathogenicity factors are VacA and the cytotoxin-associated gene pathogenicity island (*cag*PAI). VacA is an anion channel-forming cytotoxin named for its ability to fill infected cells with structures resembling swollen vacuoles (Iwamoto et al., 1999). Perhaps most importantly, it also has properties which allow the bacteria to evade the immune response; VacA can inhibit both CD8+ and CD4+ T lymphocyte and B lymphocyte proliferation, critical steps in initiation of the immune response (Torres et al., 2007). While all *H. pylori* strains express some type of VacA, it is hypothesized that bacteria which possess the more active allele combinations may be more adept at persistent colonization of their hosts (Polk and Peek, 2010). The *cag*PAI is a 40kbp fragment of DNA containing 27 potential coding regions (Censini et al., 1996) which is found in many strains of *H. pylori*. It encodes a type 4 secretion

system (T4SS) and the virulence factor CagA. CagA, a 125-140kDa protein, is injected into *H. pylori*-infected host epithelial cells where it is often activated by tyrosine phosphorylation by the host src kinase and targets host proteins to modify cellular responses (Hatakeyama, 2008; Peek, 2005). Overwhelming evidence ties the presence of CagA to an increased risk of non-cardia gastric carcinoma over the risk caused by infection with CagA-negative *H. pylori* (Kamangar et al., 2006; Palli et al., 2007; Parsonnet et al., 1997; Sasazuki et al., 2006). The mechanisms of CagA pathogenicity will be discussed in more detail below.

H. pylori has been classified as a Group I carcinogen by the International Agency for Research on Cancer since 1994. Numerous studies have been done to elicit the link between *H. pylori* infection and gastric cancer. A pooled reanalysis by the Helicobacter and Cancer Collaborative Group combined results from twelve studies and found that the matched odds ratio for the association of *H. pylori* infection and non-cardia cancer was 2.97 (95% CI: 2.34-3.77) (Helicobacter and Cancer Collaborative Group, 2001). Both intestinal and diffuse types of gastric carcinomas are associated with *H. pylori* infection, but only cancers found distal to the cardia have been linked to *H. pylori* infection. Besides gastric adenocarcinoma, infection with the bacterium has also been linked to gastric ulcers, gastritis, and MALT lymphoma. Worldwide, gastric cancer is the fourth most diagnosed cancer and the second most common cause of cancer-related death, and *H. pylori* is the causative agent in approximately 63% of these cancers.

Many proposed mechanisms for the pathogenicity of *H. pylori* exist, including changes in gene expression, infection-induced cell proliferation, epithelial cell elongation and loss of polarity, degradation of cell–cell junctions, and decreased gastric acid secretion. Recently, *H. pylori* was shown to inactivate the gastric tumor suppressor RUNX3 by two mechanisms: proteasome-mediated degradation (Tsang et al., 2010) induced by the *H. pylori* virulence factor

CagA, and gene silencing via promoter hypermethylation of *runx3* (Kitajima et al., 2008). CagA is also known to bind E-cadherin, thus interfering with the normal regulation of β-catenin, a protein whose dysregulation has been shown to cause transdifferentiation of numerous cell lineages and increased proliferation (Murata-Kamiya et al., 2007). Additionally, CagA inhibits the polarity regulator PAR1b/MARK2 kinase, which causes both loss of polarity and plays a role in the disruption of normal epithelial architecture (Saadat et al., 2007). CagA also binds and activates SHP-2 phosphatase, disrupting cell focal adhesions (Tsutsumi et al., 2006). *H. pylori* infection can also inhibit gastric acid secretion; virulence factor VacA prevents proton pump-containing vesicles from binding to the apical membrane of parietal cells (Wang et al., 2008), and *cag*PAI genes can inhibit proton pump gene expression in parietal cells (Saha et al., 2010). Hypochlorhydria can lead to gastric colonization by other, more potent bacterial inducers of inflammation, and this increased inflammation leads to development of adenocarcinoma in mice (Sanduleanu et al., 2001; Zavros et al., 2002). All together, these suggest many different mechanisms by which *H. pylori* infection may lead to a host of changes in the stomach epithelia leading eventually to the genesis of cancer. However, one of the most important mechanisms of carcinogenesis is likely the induction of chronic inflammation by *H. pylori*.

1.2 Inflammation and cancer

Many factors are known to contribute to the development of cancer, and gastric cancer development is no different. Chronic infections, tobacco smoke, diet, and obesity are all major factors which play into the development of cancer (Aggarwal et al., 2009; Wroblewski et al., 2010). There is a single mechanism which underlies all of these risk factors: inflammation (Aggarwal et al., 2009).

Hepatitis B or C virus infections, given their names because of their ability to cause chronic liver inflammation, can lead to hepatocellular carcinoma (Bouvard et al., 2009). Infections with many types of human papillomaviruses are recognized as key risk factors for cervical cancer, and the inflammation caused by persistent infection acts as a "cofactor" in carcinogenesis (Boccardo et al., 2010). A protein product of human T-cell leukemia/lymphotrophic virus type 1 (HTLV-1) "hijacks" host inflammation-regulating pathways to induce inflammation and transform T-cells, leading to the development of adult T-cell leukemia (Sun and Ballard, 1999).

There are more proposed mechanisms by which inflammation can lead to tumor initiation and promotion than can possibly be covered here, but a brief overview will be given. Tumor initiation generally requires a collection of four or more oncogenic mutations in a single cell which can transmit the mutations on to its progeny (Grivennikov et al., 2010). Inflammation can effect both increased mutation rates and upregulated cell growth. For example, inflammatory cytokines such as TNF-α, produced in response to a wide variety of stimuli, can induce production of oxidant-generating enzymes in immune cells such as macrophages and neutrophils. These enzymes cause the accumulation of reactive oxygen and nitrogen species (ROS and RNS, respectively) which are meant to attack and clear the offending microbes; however, ROS/RNS also cause single- and double-stranded DNA breaks, point and frameshift mutations and chromosomal abnormalities (Ohshima et al., 2003). One of the oncogenes noted to be affected by ROS/RNS-induced DNA mutations is p53 (Souici et al., 2000), which represents another mechanism by which inflammation can increase mutation rates: dysregulation of DNA-damage response pathways. In addition to p53 inactivation, inflammation can also upregulate the expression of activation-induced cytidine deaminase (AID), an enzyme which is normally tightly

regulated because of its function in somatic hypermutation (Okazaki et al., 2007). AID is also upregulated by inflammatory cytokines, and its overexpression contributes to the development of lymphoma, liver and gastric cancers (Grivennikov et al., 2010).

Inflammation can also induce stem cell-like properties in cells, increasing the chances of accruing the necessary mutations or allowing the growth of tumor progenitors. For example, pro-proliferation factors such as signal transducer and activator of transcription 3 (STAT3), nuclear factor-κB (NF-κB) and β-catenin are all highly activated in response to many inflammatory pathways. STAT3 is activated by pro-inflammatory cytokines such as IL-6 and IL-23, and it establishes its own feed-forward loop by transcribing more cytokines. Other targets of STAT3 include genes involved in anti-apoptosis, pro-proliferation and the epithelial-mesenchymal transition (Masuda et al., 2010). STAT3 is recognized as a *bona fide* oncogene (Bromberg et al., 1999), and its role in maintaining DNA-damaged stem cells has lead to the hypothesis that it may be instrumental in the development of cancer stem cells (Masuda et al., 2010). Constitutive NF-κB activity, often induced by chronic inflammation, has long been seen as a precursor to malignancy, and is found in many different types of cancers (Rayet and Gelinas, 1999). NF-κB activation in colonic crypts can also upregulate β-catenin activity, a transcription factor which targets genes such as cyclin D1 and c-myc to regulate proliferation. In the study by Umar et al, activation of this pathway leads to hyperplasia in the crypts of mice (Umar et al., 2009), and β-catenin is hypothesized to induce a "progenitor phenotype" in colon cancer cells (van de Wetering et al., 2002).

1.3 Inflammation induced by *Helicobacter pylori*

H. pylori infection leads to inflammation through a variety of pathways, induced both in the gastric epithelial cells which they first contact and in circulating immune cells recruited to the site of infection. Inflammatory molecules found to be upregulated in the stomachs of *H. pylori*-infected patients include IL-1, IL-6, IL-8, TNF-α, and RANTES, which are all regulated by many different pathways (Gionchetti et al., 1994; Noach et al., 1994; Shimoyama et al., 1998). One common pathway may be through the transcription factor NF-κB (Baeuerle and Henkel, 1994); however, much of the literature describes only the induction of one or more of these cytokines, particularly IL-8.

H. pylori utilizes many different mechanisms for the induction of pro-inflammatory cytokines. Peptidoglycan can enter host epithelial cells via the *cag*PAI-encoded T4SS and stimulate the intracellular pathogen-recognition receptor Nod1, which in turn signals to activate NF-κB and AP-1 for the induction of cytokines such as IL-8 (Allison et al., 2009; Viala et al., 2004). OipA and BabA, two outer membrane adhesion proteins, were shown by knock-out studies to be important in the induction of IL6, IL-8 and IL-11 production, though the mechanism is as yet unclear (Sugimoto et al., 2011; Yamaoka et al., 2000). VacA appears to be primarily for immunosuppression of T cell activation, allowing persistence of the bacterial infection, but evidence also shows that the toxin can induce some NF-κB activity within targeted T cells (Takeshima et al., 2009). The virulence factor CagA has been studied extensively, especially its role in *H. pylori*-induced inflammation. More introduction will be given elsewhere; briefly, bacteria which translocate CagA into host cells induce higher levels of IL-8 production (Backert et al., 2004), and activate NF-κB, AP-1 and NFAT (Meyer-ter-Vehn et al., 2000; Naumann et al., 1999; Yokoyama et al., 2005).

Besides activating cytokine release, *H. pylori* also stimulates ROS/RNS production. *H. pylori* infection activates AP-1 to induce COX-2 and iNOS transcription, which in turn produce prostaglandin E2 and nitrite (Cho et al., 2010). Additionally, VacA was shown to induce ROS production and mitochondrial DNA mutation in gastric epithelial cells (Huang et al., 2011). *H. pylori* sonicates were observed to induce oxidative bursts from neutrophils and monocytes in culture (Hansen et al., 1999)via a mechanism involving the virulence factor *H. pylori* neutrophil-activating protein (HP-NAP), a secreted protein which can pass through the epithelial layer into the lamina propria to attract leukocytes (D'Elios et al., 2007). HP-NAP induces ROS release from neutrophils (Evans et al., 1995) and causes the recruitment of other leukocytes to the site of infection by stimulating the production of chemokines such as CXCL8, CCL3 and CCL4 (Polenghi et al., 2007). Mast cells, recruited by these chemokines, are stimulated to degranulate by HP-NAP, likely through a Gi protein-coupled receptor(Montemurro et al., 2002). Altogether, these mechanisms create an inflammatory environment conducive to the initiation and progression of cancer.

1.4 NF-κB: master regulator of inflammation

NF-κB was discovered 25 years ago and is now one of the most extensively studied transcription factors. In mammals, the NF-κB/Rel family consists of seven proteins, including RelA/p65, c-Rel, RelB, p100, p52, p105 and p50. Each protein contains a Rel homology domain (RHD) within the N-terminus and can form homo- or heterodimers through the RHD (Ghosh et al., 1998). It is a master regulator of many cellular processes important in carcinogenesis, including inflammation, transformation, proliferation, angiogenesis and metastasis. Dysregulated NF-κB is important in the pathophysiology of inflammatory diseases such as asthma, rheumatoid

arthritis, and systemic lupus erythematosus (Ghosh and Karin, 2002; Sethi et al., 2008). Additionally, NF-κB is constitutively active in most human cancers, a phenotype created by persistent stimulation, such as through chronic infection, or via mutation leading to pathway activation (Chaturvedi et al., 2011).

There are many pathways that lead to the activation of NF-κB, and many stimuli, including lipopolysaccharide (LPS), peptidoglycan and TNF-α(Hayden and Ghosh, 2008), which activate the canonical pathway, and lymphotoxin, CD40 ligand and RANK ligand, which primarily activate the non-canonical pathway(Sun, 2011). In the canonical pathway, the binding of these ligands to their receptors leads to the activation of signaling pathways which converge upon the phosphorylation and activation of the IκB kinase (IKK) complex, which consists of IKKα, IKKβ and NEMO/IKKγ. This kinase complex in turn phosphorylates and induces the degradation of the inhibitor of κB, IκBα, which in unstimulated conditions binds and sequesters NF-κB within the cytoplasm. NF-κB, a heterodimer of p50 and RelA/p65, is released and moves into the nucleus, where it transactivates its target genes involved in inflammation, cell growth and survival (Chen and Greene, 2004). Posttranslational modifications, including methylation, acetylation and phosphorylation, are added to p65 in particular to modulate the activity of the transcription factor (Yang et al., 2009c), and both ubiquitination of p65 and resynthesis of IκBα lead to abrogation of NF-κB nuclear activity(Yang et al., 2009b). In the non-canonical pathway, signaling from receptors activates NF-κB-inducing kinase (NIK), which activates IKKα. Together, these kinases induce proteasomal processing of p100 to the active p52, which pairs with RelB to form the transcriptionally active complex which activates target genes involved in lymphoid organogenesis, B cell survival and maturation, and bone metabolism(Sun, 2011).

Due to the important role of NF-κB in inflammation-related diseases and cancer, how *H. pylori* activates NF-κB has been a long-standing question. While a number of studies have focused primarily on the importance of different *H. pylori* virulence factors in activation of inflammation in general, very little is understood with regards to how *H. pylori* specifically induces the NF-κB pathway. Here, I investigate the mechanisms behind *H. pylori* virulence factor-induced activation of NF-κB.

CHAPTER 2

HELICOBACTER PYLORI ACTIVATES NF-κB IN A CagA-DEPENDENT MANNER

2.1 Introduction

H. pylori colonize the mucosal layer of the stomach, attaching themselves to the gastric epithelium via various bacterial adhesins and epithelial receptors (Rubinsztein-Dunlop et al., 2005). The epithelium is therefore the first point of contact for the bacteria in the host, and the bacteria rarely invade any farther than the epithelial layer. Immune cells attracted to the site of infection by cytokines released from the epithelial cells also respond to *H. pylori*. This further amplifies the immune response from the infection and the resulting inflammation damages the mucosal layer, which can result in ulcers, gastritis and adenocarcinoma (Ibraghimov and Pappo, 2000). Although *H. pylori* effects responses from both epithelial cells and immune cells such as monocytes and lymphocytes, the *H. pylori*-induced inflammatory response generated by gastric epithelial cells is the most well studied and likely the most important for understanding the development of gastric adenocarcinoma.

Infection with *cag*PAI-positive *H. pylori* is associated with more severe outcomes in the inflammation-linked illnesses mentioned as compared to infection with *cag*PAI-negative *H. pylori* (Blaser et al., 1995; Kuipers et al., 1995). The approximately 40 kb cytotoxin-associated gene (*cag*) pathogenicity island contains genes which encode a type 4 secretion system (T4SS), a syringe-like projection which protrudes from the cell wall of the bacterium and makes contact with the host cell's membrane, and a pathogenicity factor called cytotoxin-associated gene A, or CagA(Censini et al., 1996). A protein ranging in size from 120–145 kDa, CagA is injected into

the host epithelial cells via the T4SS, where it acts on a number of different signaling pathways leading to inflammation and cell scattering (Hatakeyama, 2004).

A role for CagA in the activation of NF-κB and the production of IL-8 has been suggested by many studies. For example, using an interleukin-8 (IL-8) promoter-reporter assay, Sharma et al. showed the requirement of CagA for NF-κB activation (Sharma et al., 1998). It was also shown that ectopically expressed CagA induced NF-κB translocation into the nucleus and also induced IL-8 production in gastric epithelial cells (Brandt et al., 2005; Kim et al., 2006). Additionally, NF-κB activation and inflammation was markedly less in the gastric antra of Mongolian gerbils infected with *cagA*-deficient *H. pylori* as compared to infection with wild-type *H. pylori* (Shibata et al., 2006). However, the exact function of CagA in NF-κB activation is still unclear. Here, I demonstrate a clear dependence on the presence of CagA for NF-κB activation by *H. pylori* in gastric epithelial cells.

2.2 Materials and Methods

Cell lines, *H. pylori* culture and infection. Human AGS gastric adenocarcinoma and HEK293T cells were cultured in DMEM supplemented with 10% fetal bovine serum. *H. pylori* G27, NCTC11637 and 7.13 strains and their *cagA*-deficient mutants were cultured in bisulphite-free *Brucella* broth on agar media containing Ham's F-12 medium supplemented with 10% fetal bovine serum and 5 μg/ml vancomycin at 37°C in the presence of 10% CO_2. *H. pylori* was added to AGS cells for infection at a multiplicity of infection of 50–100. All wild-type *H. pylori* and the corresponding *cagA*-deficient strains have similar abilities to adhere to the cells.

Antibodies. Normal rabbit IgG, antibodies against CagA, IκBα, and RelA were from Santa Cruz Biotechnology. Phospho-S536 RelA was from Cell Signaling.

Immunoprecipitation, immunoblotting analysis and EMSA. Lysates from HEK293T or AGS cells were collected, cleared and denatured in SDS sample buffer. Lysates were separated by SDS-PAGE (12%), transferred to nitrocellulose transfer membranes and immunoblotted with various antibodies followed by visualization of the immunoreactive proteins by ECL (Amersham). For electrophoretic mobility-shift assay (EMSA), whole-cell extracts were collected from infected cells by freeze–thaw lysis. Cells were washed once with cold PBS, then 0.5 mL cold PBS was added to each well and cells were harvested with a plastic scraper. Cells were collected by centrifugation, then cell pellets were lysed with whole cell extract buffer (20mM Hepes pH 7.0, 0.4M NaCl, 0.1% NP-40, 25% glycerol, 1mM EDTA, 2.5mM dithiothreitol, and 1mM phenylmethylsulfonyl fluoride). Cell pellets were mixed by pipetting and incubated on ice for 30 min, then vortexed for 30 s. Lysates were frozen in liquid nitrogen, thawed on ice, and vortexed for 1 min before centrifuging to collect supernatants. NF-κB DNA-binding activity was assessed in electrophoretic mobility shift assays (EMSA) with a ^{32}P-radiolabeled consensus κB enhancer oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) incubated with 6μg whole cell extract at 30˚C for 15 min. For supershift assay, 2μL of either anti-RelA (SC-109, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-p50 (SC-1190, Santa Cruz) was added to the mixture. Comparability of the various nuclear extracts was assessed by EMSA with a ^{32}P-radiolabeled Oct1 probe (Promega).

Quantitative real-time PCR analysis. AGS cells were infected with *H. pylori* for various times and total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Complementary DNA was synthesized using the Omniscript RT kit (Qiagen). Quantitative real-time PCR was performed using the Qiagen SYBR green PCR kit, with the aid of the 7300 Real-

time PCR system (ABI, Foster City, CA, USA). PCR primers for human β-actin, IL-8 and TNF-α were purchased from Qiagen.

Transient transfection, plasmids and luciferase reporter assay. CagA-HA and mutants have been described (Lu et al., 2008), and were a gift from Dr. M. Hatakeyama. For experiments using HEK293T cells, transfection was performed using the calcium phosphate method. For experiments using AGS cells, either calcium phosphate or Lipofectamine 2000 was used to transfect cells (Invitrogen). For luciferase assay, cells were infected with *H. pylori* 24 hours post-transfection, and then lysed in passive lysis buffer; firefly and renilla luciferase activities were measured with the dual luciferase assay system from Promega. Firefly luciferase activity was normalized to renilla luciferase activity. Results represent the average of three independent experiments ± s.d.

2.3 Results

To investigate the role of CagA in the *H. pylori*-induced inflammatory response, we compared wild-type and *cagA*-deficient *H. pylori* for their ability to induce the expression of inflammatory genes in AGS gastric epithelial cells. Infection of AGS cells with wild-type *H. pylori* strain G27, but not its *cagA*-deficient isogenic mutant, induced the expression of IL-8 and TNF-α messenger RNA (mRNA; Fig 2.1). Similar results were obtained with two other *H. pylori* strains: NCTC11637 and 7.13 (Fig 2.2). These data indicate that induction of proinflammatory genes can be generalized for most *cagA*-positive *H. pylori* strains. Further, since multiple strains and their derived mutants give similar results, the defect from the *cagA*-deficient mutants is unlikely to be due to a polar effect.

Figure 2.1

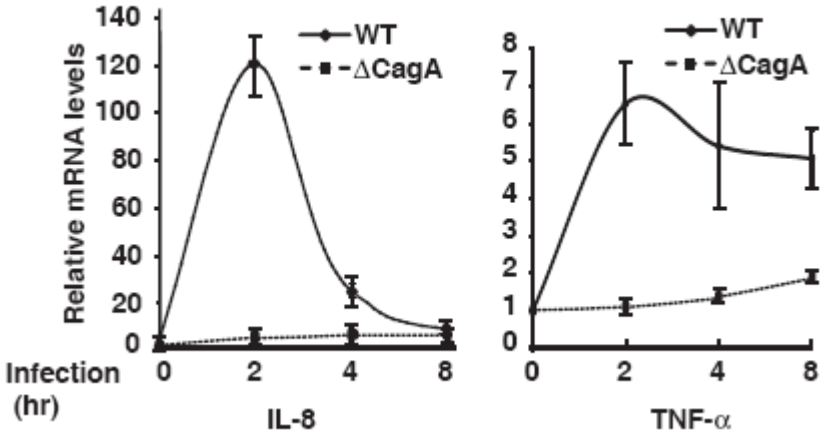

Figure 2.1. CagA is required for activation of NF-κB. AGS cells were infected with wild-type or *cagA*-deficient *H. pylori* for indicated timepoints, genes involved in the inflmmatory response were measured.

As NF-κB regulates the expression of many inflammatory cytokines, including IL-8 and TNF-α, we next investigated whether *H. pylori* infection activated NF-κB and whether this

Figure 2.2

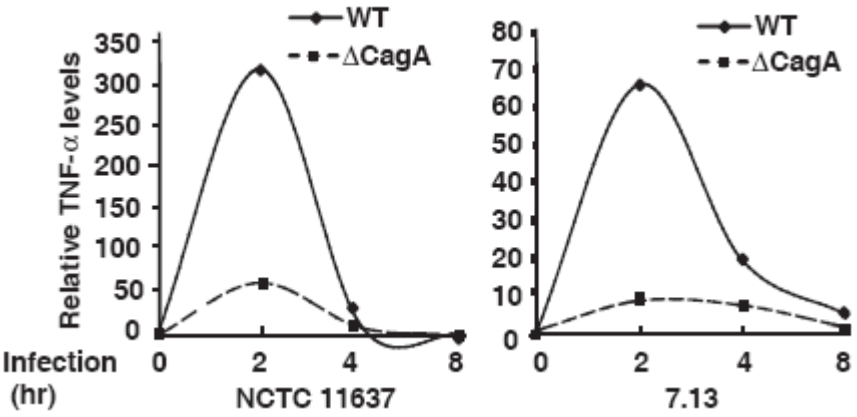

Figure 2.2. CagA is required for activation of NF-κB. AGS cells were infected with wild-type or *cagA*-deficient strains of *H. pylori* for indicated timepoints, TNF-α mRNA was measured.

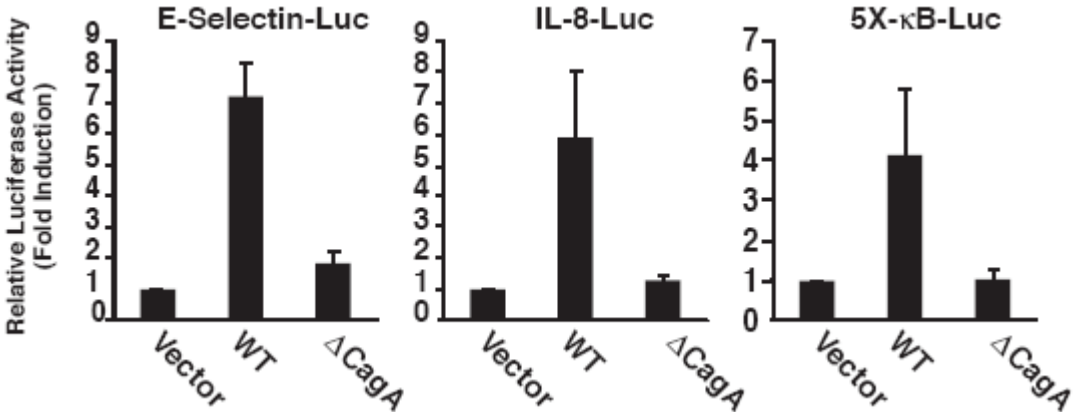

Figure 2.3. *H. pylori* activates NF-κB in a *cagA*-dependent manner. 5XκB-luciferase (5XκB-Luc), IL-8-luciferase (IL-8-luc) or E-selectin-luciferase (E-selectin-luc) reporter plasmids were transfected into AGS cells followed by infection with *H. pylori*. Luciferase activity was measured 6 h after infection.

activation was CagA-dependent. Wild-type G27 *H. pylori*, but not its *cagA*-deficient mutant, activated all three κB luciferase reporters, including two that contain natural promoters from NF-κB target genes IL-8 and E-selectin (Fig. 2.3). Furthermore, only wild-type *H. pylori*, but not the *cagA*-deficient mutant, stimulated the binding of NF-κB, a heterodimer of RelA and p50, to DNA, showing that CagA specifically activates the canonical NF-κB pathway (Fig. 2.4).

Phosphorylation and multimerization are two properties of CagA that are essential for many of its functions within host epithelial cells, including the ability to deregulate the SHP-2

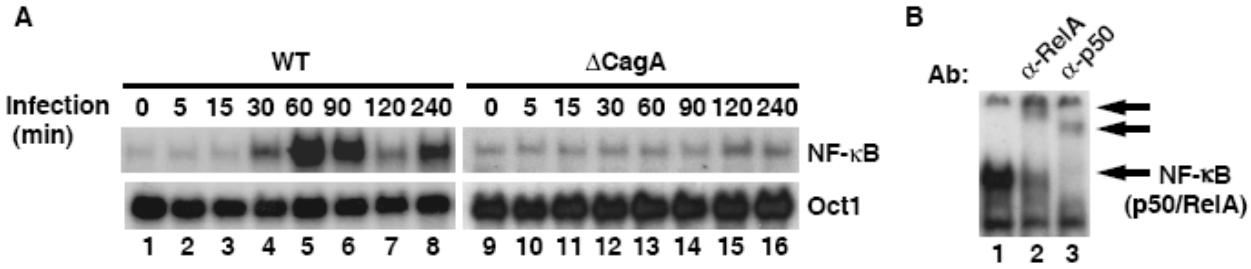

Figure 2.4. *H. pylori* stimulates binding of NF-κB to DNA. *A* EMSA was performed using whole-cell extracts from AGS cells infected with wt or ΔCagA *H. pylori* by using radiolabelled NF-κB and Oct1 probes. *B* Super-shift assay using anti-RelA or anti-p50 antibodies was performed using WT 60 min timepoint sample from Fig 2.4A. Super-shifts are indicated by arrows.

phosphatase to induce the "hummingbird" phenotype and the ability to bind the PAR1 kinase to inhibit cell-cell junction stability, respectively (Hatakeyama, 2008). To determine whether either of these modifications are necessary for CagA activation of NF-κB, wild-type, phosphorylation-deficient (PR), single EPIYA and multimerization domain-containing (ΔABCC), or multimerization-deficient (ΔABCC-16AA) CagA were ectopically expressed along with luciferase reporter and Renilla control plasmids in HEK293T cells. Though expression of CagA was low, NF-κB activity did increase over basal activity, indicating that CagA alone was sufficient for NF-κB activation (Fig. 2.5). Further, while neither the phosphorylation-deficient mutant nor the single EPIYA and multimerization domain-containing mutant showed a changed ability to activate NF-κB, multimerization-deficient CagA was not able to activate NF-κB (Fig.

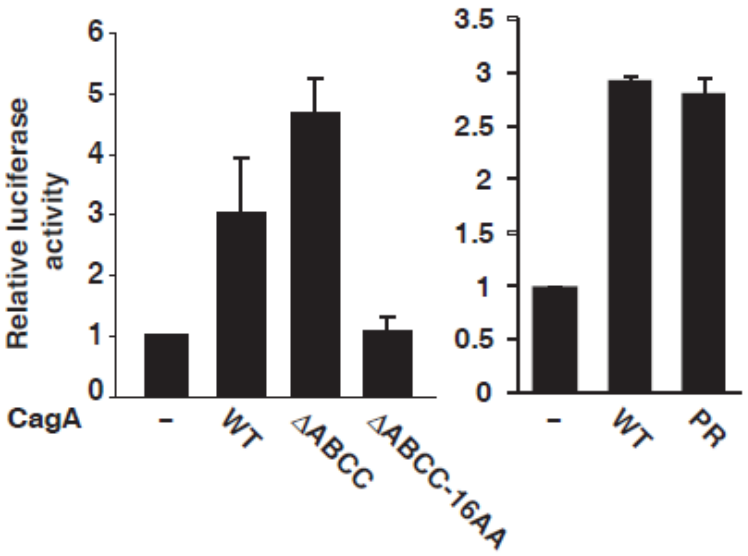

Figure 2.5. Oligomerization, but not phosphorylation, is required for activation of NF-κB by CagA. 293T cells were transfected with IL-8-luciferase, Renilla, and wild-type and various CagA-deletion mutants. 30 hours post-tranfection, cells were harvested and lysates were assayed for luciferase activity.

2.5). These data show that while phosphorylation of CagA is dispensible for NF-κB activation, multimerization is not.

To define the signaling pathway leading to the activation of NF-κB, we examined the degradation and re-synthesis of IκBα and phosphorylation of RelA in AGS cells infected with wild-type and *cagA*-deficient *H. pylori*. IκBα was degraded and resynthesized in cells infected with wild-type but not in those infected with *cagA*-deficient *H. pylori* (Fig. 2.6). In addition,

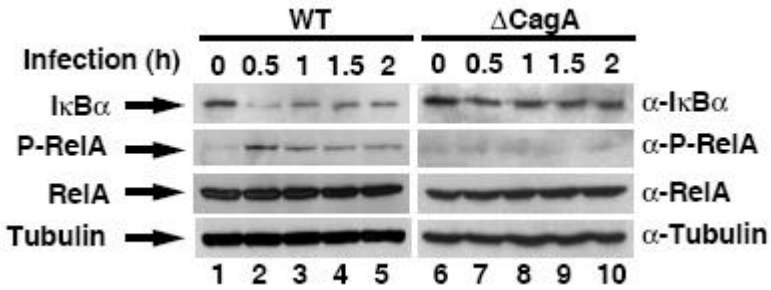

Figure 2.6. *H. pylori* activates NF-κB through the canonical pathway. Levels of IκBα, phosphorylated Ser 536 RelA, RelA, CagA and tubulin were detected in lysates from AGS cells infected with G27 wt or ΔCagA *H. pylori* by immunoblotting with the indicated antibodies.

wild-type *H. pylori*, but not the *cagA*-deficient mutant, stimulated the phosphorylation of RelA at Ser 536, which is also mediated by activated IKK (Fig. 2.6). All together, these data indicate that *H. pylori* infection stimulates CagA-dependent activation of NF-κB through the activation of IKK.

2.4 Discussion

Despite the essential role of the *cag*PAI in the activation of NF-κB and the induction of IL-8 in epithelial cells, the requirement for CagA, not just the T4SS, for the inflammatory

response has been a point of contention nearly since the discovery of the pathogenicity island. It has been reported that the T4SS, but not CagA, is required for *H. pylori*-mediated production of IL-8 since T4SS mutants failed to induce IL-8 (Naumann, 2005). However, as the T4SS translocates CagA into host cells, impaired NF-κB activation by mutants in which the T4SS is defective cannot rule out the possibility that the important component is, in fact, CagA. Another concern regarding the requirement of CagA for *H. pylori*-mediated NF-κB activation is the potential for polar effects from the deletion of the *cagA* gene, in which disruption of one gene causes disruption of genes downstream within the same operon (Fischer et al., 2001). Nevertheless, as *cagA* is monocistronic and transcribed in a different direction than other genes comprising the *cag*PAI (Censini et al., 1996), it is unlikely that the *H. pylori* isogenic *cagA* mutant strains are exerting polar effects on other genes within the *cag*PAI. Concordantly, no polar effects have been reported for the *cagA*-deficient *H. pylori* strains in the literature.

While CagA has clearly been shown to be essential for the activation of NF-κB, it must be noted that this requirement could be *H. pylori* strain-specific. The considerable variation in the sequences of CagA from different *H. pylori* strains led to the designation of "Western" and "Eastern" strains based on the sequences surrounding the phosphorylation domains in the C terminus of the protein. These differences correlate with the ability of the CagA protein to associate with SHP-2 and induce the "hummingbird" phenotype (Higashi et al., 2002). Sequence variations within the CagA multimerization domain influence cell-cell junctions and cell polarity along with association with SHP-2 (Lu et al., 2008). In addition to affecting cell and epithelial morphologies, strain variations have also been linked to variations in IL-8 production. Exchanging *cagA* genes allows low IL-8-inducing *H. pylori* strains to be converted into high inducing strains and vice versa (Brandt et al., 2005). We hypothesize that these effects may be

caused by increased or decreased abilities of these CagA proteins to bind and activate upstream signaling components, such as TAK1, influencing the downstream activity of NF-κB. However, the sequences required for CagA to induce NF-κB activation remain elusive and need further investigation.

Additionally, the requirement for CagA in the activation of NF-κB could also be cell-type specific. CagA seems to not be essential for *H. pylori*-induced activation of NF-κB in cells other than gastric epithelial cells. In macrophages, *H. pylori* activates NF-κB via TLR2 (for induction of IL-6 and IL-1β) and TLR4 (for induction of IL-12, IL-10 and IL-8) (Maeda et al., 2001; Obonyo et al., 2007). Findings similar to those of the activation in macrophages were reported in lymphocytes by Ohmae et al. In human B lymphocytes, *H. pylori* activates not only the NF-κB classical pathway, but also the non-canonical pathway, which involves the processing of p100, reportedly by TLR4 recognition of *H. pylori* LPS (Ohmae et al., 2005).

CHAPTER 3

TAK1 UBIQUITINATION AND ACTIVATION IS ENHANCED BY HELICOBACTER PYLORI CagA

3.1 Introduction

Many pathways activate NF-κB, including both the classical and non-classical pathways, and there are a great number of signaling molecules involved in all of the pathways. How CagA is able to activate the canonical NF-κB pathway is an important question for the understanding of *H. pylori*-induced inflammation, as well as for understanding the mechanisms which pathogens may use to dysregulate a system. One critical signal transducer which has recently been found to be targeted by virulence factors of other pathogens, such as human T-lymphotrophic virus-1 protein TAX and herpes simplex virus-1 ICP0 (Wu and Sun, 2007, Diao et al., 2005), is transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1).

TAK1, a mitogen-activated protein kinase kinase kinase (MAPKKK) family member which phosphorylates serine/threonine, was initially found to be activated by TGF-β and bone morphogenetic protein (Yamaguchi et al., 1995). Besides members of the TGF-β family, TAK1 can also be activated in response to IL-1, TNF-α, and Toll-like receptor ligands. Its targets include MKK3/6, IKKβ, AMP-activated protein kinase, LKB1, and p38 (Wang et al., 2001; Witczak et al., 2008; Xie et al., 2006; Yamaguchi et al., 1995), which can regulate transcription factors such as AP-1, NF-κB and p53 (Oeckinghaus et al., 2011; Yaswen and Campisi, 2007). The myriad targets of TAK1 give it a pivotal role in the regulation of immunity, inflammation, differentiation and growth, and cell survival.

Though the activation of IKK by TAK1 is not clearly understood, emerging evidence indicates that it is regulated by tumor necrosis factor receptor-associated factor 6 (TRAF6)-mediated, lysine 63 (K63)-linked ubiquitination of TRAF6, TAB2/3, and NF-κB essential modulator (NEMO; Adhikari et al., 2007). Ubiquitin is a small protein which can be catalytically added to lysines of targeted proteins by being activated by one of two E1s, transferred to one of around thirty different E2s, and then added to the target selectively by one of many hundred E3 ligases. One or a progressively linked chain of ubiquitin molecules, linked from one of seven internal lysines to the C-terminus of the next molecule, may be added to a target. K48-linked and K63-linked ubiquitin chains are the two most prevalent types of linkages; K48-linked ubiquitination functions to signal proteasomal degradation, while K63-linked ubiquitination functions as a scaffold to bring together proteins in a signaling pathway (Chen, 2005)(Chen and Greene, 2005; Chen, 2005).

TAB2/3 interact with TAK1 constitutively, and their interaction with K63-linked polyubiquitin chains is required for the activation of TAK1 (Kanayama et al., 2004). NEMO ubiquitination is not well understood, though it appears to be required for NF-κB activity in response to TLR activation (Ni et al., 2008); additionally, NEMO has ubiquitin binding domains which may be required for interacting with the TAK1 complex (Ea et al., 2006). Recent studies also show that TAK1 is polyubiquitinated by TRAF6 in response to TGF-β, and that K63-linked ubiquitination is required for TGF-β-induced activation of p38/JNK and AP-1 (Sorrentino et al., 2008), indicating that TAK1 ubiquitination might also be crucial for the activation of IKK and NF-κB.

Many of CagA's targets are found near the membrane, like many receptors and receptor-bound intermediates which can trigger TAK1 activation. The interaction of CagA with one membrane protein, the hepatocyte growth factor receptor Met, leads to the activation of another important kinase, Akt (Suzuki et al., 2009). Combined with the evidence that *cagA*-positive *H. pylori* activates the IKK complex, we were thus interested in determining whether *H. pylori* CagA might cause the activation of TAK1.

3.2 Materials and Methods

Cell lines, *H. pylori* culture and infection. Human AGS gastric adenocarcinoma, HEK293T and MEF cells were cultured in DMEM supplemented with 10% fetal bovine serum. *H. pylori* G27 was cultured in bisulphite-free *Brucella* broth on agar media containing Ham's F-12 medium supplemented with 10% fetal bovine serum and 5 μg/ml vancomycin at 37°C in the presence of 10% CO_2. *H. pylori* was added to AGS or MEF cells for infection at a multiplicity of infection of 50–100.

Plasmids. CagA was amplified by PCR from the genomic DNA of *H. pylori* strain G27 and subcloned into the pcDNA3.1-Flag or pcDNA3.1-T7 vector. HA-TAK1 was received as a gift from Dr. J. Ninomiya-Tsuji and Flag-TAK1 was received as a gift from Dr. X. Lin. His-MKK6 (K82A) was received as a gift from Dr. Z. J. Chen.

Antibodies. Normal rabbit IgG, antibodies against CagA, TAK1, IKK1, IκBα, RelA, Flag, HA, His and ubiquitin were from Santa Cruz Bio. Anti-T7 antibody was from Covance. Phospho-S536 RelA, phospho-IκBα (S32/26) and phospho-TAK1 (T187) were from Cell Signaling.

TAK1 siRNA knockdown and rescue. TAK1 siRNA from Ambion was transfected into AGS cells using Lipofectamine 2000 according to the manufacturer's protocol. 36 hrs post-

transfection, cells were split, and 36 hr later were infected with *H. pylori*. For rescue, cells were transfected with 40ng Flag-TAK1 per well of a 12-well plate using Lipofectamine 2000 24 hrs before infection.

Immunoprecipitation, immunoblotting analysis and EMSA. Immunoprecipitation, immunoblotting analysis and EMSA. Lysates were immunoprecipitated for 2 hours at 4°C using 20 μL of a slurry containing 50% anti-T7-conjugated or anti-Flag-conjugated agarose beads (Novagen or Sigma), or with 5 μL of various antibodies and 20 μL Protein A agarose beads (Sigma). These immunoprecipitates were washed three times in lysis buffer. Lysates from HEK293T or AGS cells were collected, cleared and denatured in SDS sample buffer. Lysates or immunoprecipitates were separated by SDS-PAGE (10-13%), transferred to nitrocellulose transfer membranes and immunoblotted with various antibodies followed by visualization of the immunoreactive proteins by ECL (Amersham). For electrophoretic mobility-shift assay (EMSA), whole-cell extracts were collected from infected cells by freeze–thaw lysis. Cells were washed once with cold PBS, then 0.5 mL cold PBS was added to each well and cells were harvested with a plastic scraper. Cells were collected by centrifugation, then cell pellets were lysed with whole cell extract buffer (20mM Hepes pH 7.0, 0.4M NaCl, 0.1% NP-40, 25% glycerol, 1mM EDTA, 2.5mM dithiothreitol, and 1mM phenylmethylsulfonyl fluoride). Cell pellets were mixed by pipetting and incubated on ice for 30 min, then vortexed for 30 s. Lysates were frozen in liquid nitrogen, thawed on ice, and vortexed for 1 min before centrifuging to collect supernatants. NF-κB DNA-binding activity was assessed in electrophoretic mobility shift assays (EMSA) with a ^{32}P-radiolabeled consensus κB enhancer oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) incubated with 6μg whole cell extract at 30˚C for 15 min. For supershift assay, 2μL of either anti-RelA (SC-109, Santa Cruz Biotechnology, Santa

Cruz, CA) or anti-p50 (SC-1190, Santa Cruz) was added to the mixture. Comparability of the various nuclear extracts was assessed by EMSA with a ^{32}P-radiolabeled Oct1 probe (Promega).

Quantitative real-time PCR analysis. AGS or MEF cell lines were infected with *H. pylori* for various times and total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Complementary DNA was synthesized using the Omniscript RT kit (Qiagen). Quantitative real-time PCR was performed using the Qiagen SYBR green PCR kit, with the aid of the 7300 Real-time PCR system (ABI, Foster City, CA, USA). PCR primers for human β-actin, IL-8 and TNF-α, as well as mouse β-actin, E-selectin, IL-6 and TNF-α, were purchased from Qiagen.

Transient transfection and luciferase reporter assay. For experiments using HEK293T cells, transfection was performed using the calcium phosphate method. For experiments using AGS cells, either calcium phosphate or Lipofectamine 2000 was used to transfect cells (Invitrogen). For luciferase assay, cells were lysed in passive lysis buffer 30 hours post-transfection; firefly and renilla luciferase activities were measured with the dual luciferase assay system from Promega. Firefly luciferase activity was normalized to renilla luciferase activity. Results represent the average of three independent experiments ± s.d.

3.3 Results

To better understand the way in which *H. pylori*-induces activation of IKK and NF-κB, we investigated the role of TAK1. To do so, we first utilized mouse embryonic fibroblasts (MEFs) from paired wild-type and TAK1-deficient (TAK1-/-) mice. When these MEFs were

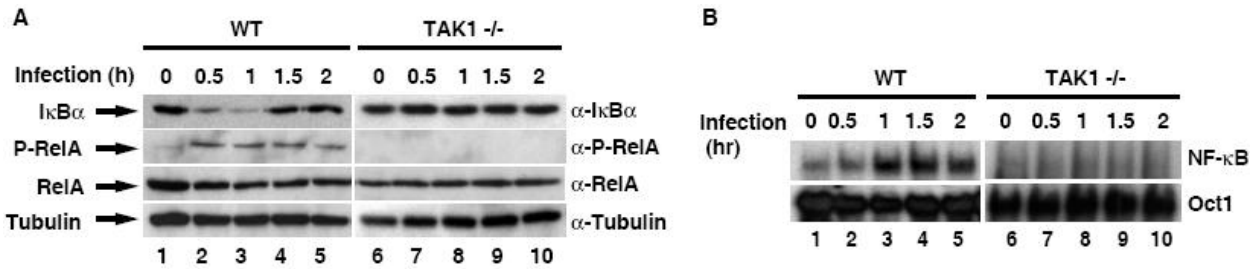

Figure 3.1. *H. pylori* activation of NF-κB requires TAK1. *A* Levels of IκBα, phosphorylated Ser 536 RelA, RelA, TAK1 and tubulin were detected in the lysates from wt or TAK1-/- MEFs infected with *H. pylori*. *B* EMSA was performed using whole-cell extracts from wt or TAK1-/- MEFs infected with *H. pylori*, as in Fig 3.1A.

infected with *H. pylori*, NF-κB activation was induced in wild-type MEFs but not in TAK1-/- MEFs, as seen by evaluating IκBα degradation and resynthesis and phosphorylation of Ser 536 of RelA (Fig 3.1A). Furthermore, *H. pylori* stimulated the DNA-binding activity of NF-κB in wild-type but not in TAK1-/- MEFs (Fig 3.1B). These data indicate that TAK1 is essential for the activation of IKK and NF-κB after *H. pylori* infection. Consistently, infection of wild-type MEFs, but not TAK1-/- MEFs, with *H. pylori* stimulated mRNA expression of TNF-α, IL-6 and E-selectin—three NF-κB target genes (Fig. 3.2).

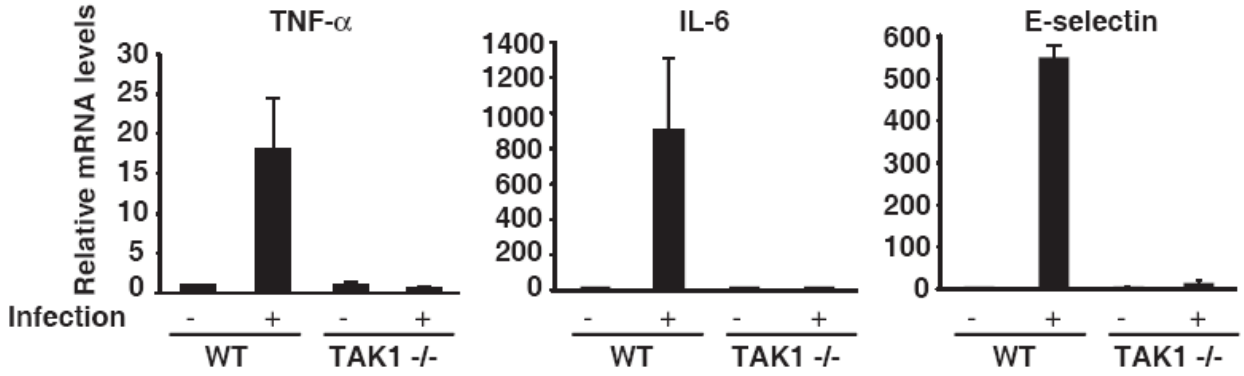

Figure 3.2. TAK1 is required for activation of NF-κB by *H. pylori* in MEFs. WT and TAK1-/- MEFs were uninfected or infected for 8 hrs with *H. pylori*. Quantitative RT-PCR was performed to analyze the *H. pylori*-induced expression of NF-κB target genes.

Since MEF cells may not accurately represent the gastric epithelium's response to *H. pylori*, we sought to determine whether TAK1 might play a role in *H. pylori*-induced NF-κB

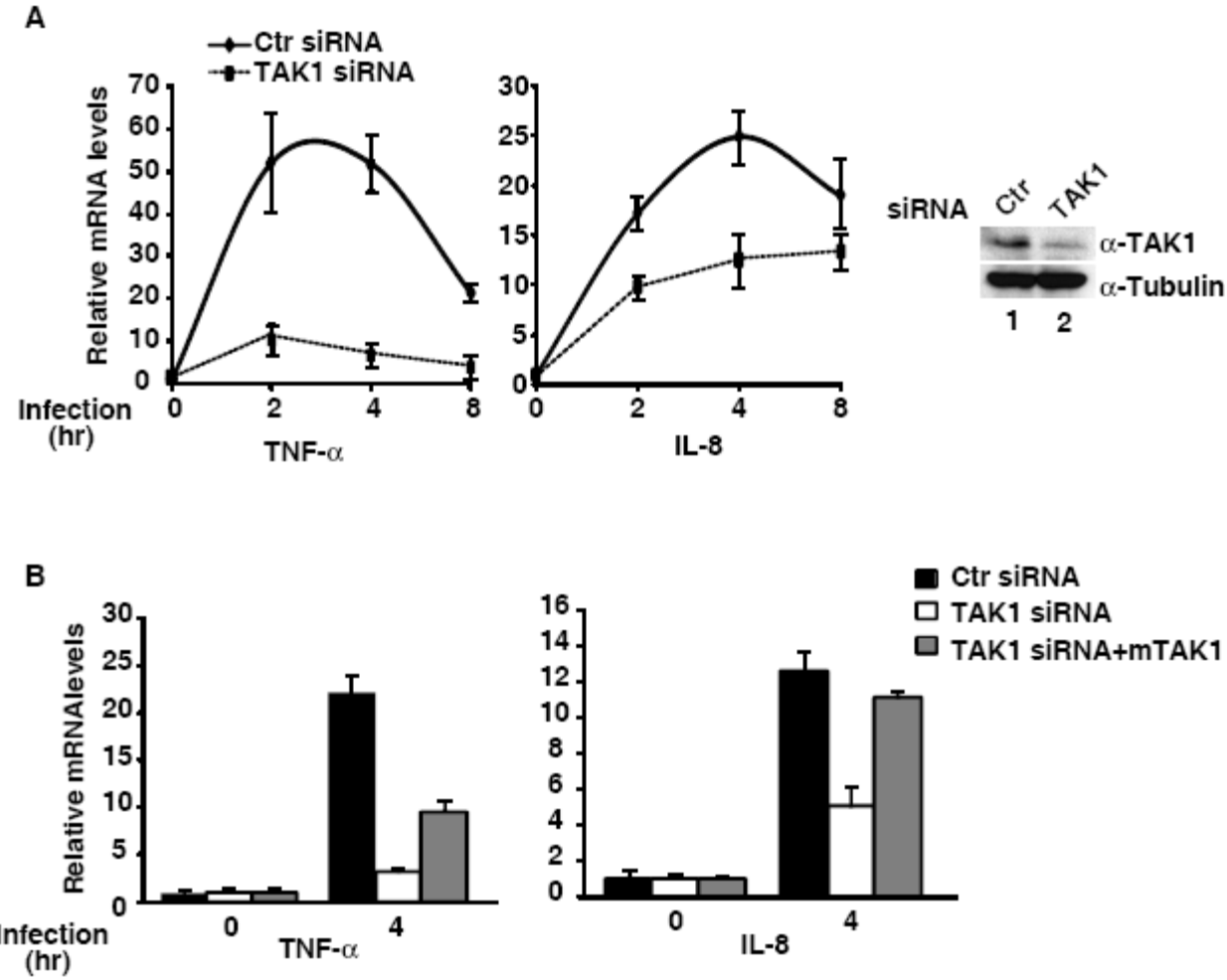

Figure 3.3. TAK1 is required for activation of NF-κB by *H. pylori* in gastric epithelial cells. *A* AGS cells transfected with control or TAK1 siRNA were infected with *H. pylori* and RT–PCR was performed to analyze NF-κB target gene expression. Levels of TAK1 and tubulin are shown in the right panels. *B* AGS cells transfected with control siRNA or TAK1 siRNA were reconstituted with mouse TAK1 (mTAK1), followed by infection for 4 h with *H. pylori*. Gene expression was analysed as shown in Fig. 3.3A.

activation in a more physiologically-relevant model. We therefore used short interfering RNA (siRNA) to knock down the expression of TAK1 in AGS cells. Depletion of TAK1 by siRNA impaired the *H. pylori*-induced mRNA expression of TNF-α and IL-8 (Fig 3.3A). The partial

decrease in expression of TNF-α and IL-8 mRNA in TAK1-knockdown cells (compared with TAK1-/- MEFs) probably reflects the incomplete depletion of TAK1 by siRNA in AGS cells (with approximately 80% knockdown efficiency; Fig 3.3A). To confirm that the impaired activation of NF-κB-driven transcription was indeed a result of depletion of TAK1 rather than a secondary effect from the siRNA, siRNA-resistant mouse TAK1 was reintroduced into the knockdown cells (Fig. 3.3B). The impaired expression of TNF-α and IL-8 was "rescued" by reintroduction of TAK1. These data confirm that TAK1 is essential for *H. pylori*-induced activation of NF-κB.

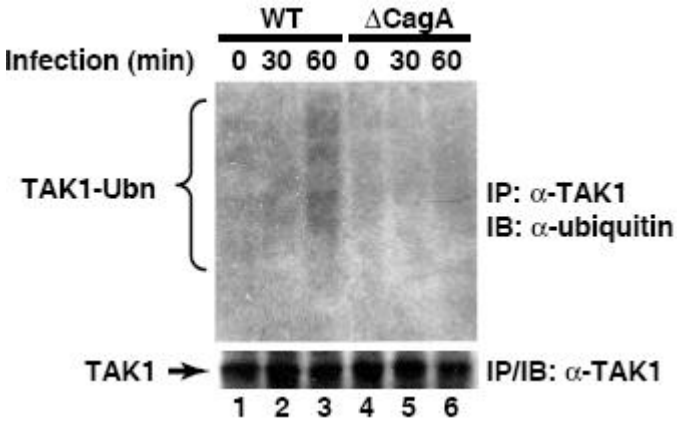

Figure 3.4. *H. pylori* infection causes ubiquitiniation of TAK1. AGS cells were infected with wild-type or Δ*cagA H. pylori*, TAK1 was immunoprecipitated and immunoblotted for ubiquitin.

As K63-linked polyubiquitination is important for the activation of TAK1 and IKK (Sorrentino et al., 2008; Wang et al., 2001), and TAK1 is essential for *H. pylori*-induced activation of NF-κB (Figs. 3.1-3.3), we next examined whether *H. pylori* stimulated polyubiquitination of TAK1. Infection of AGS cells with wild-type, but not *cagA*-deficient, *H. pylori* induced polyubiquitination of endogenous TAK1 (Fig. 3.4), indicating that CagA is crucial for *H. pylori*-induced polyubiquitination of TAK1. Next, we determined the potential

effect of CagA on the ubiquitination of TAK1. TAK1 is modestly ubiquitinated in the presence of ubiquitin, and this ubiquitination is markedly enhanced by co-transfection of CagA (Fig. 3.5A). To ensure that the ubiquitination observed was directly bound to TAK1 and not a co-immunoprecipitated partner, the ubiquitination experiment was repeated using denaturing conditions designed to separate TAK1 from any non-covalently bound partners prior to immunoprecipitation. Similar results were obtained under these conditions, indicating that while

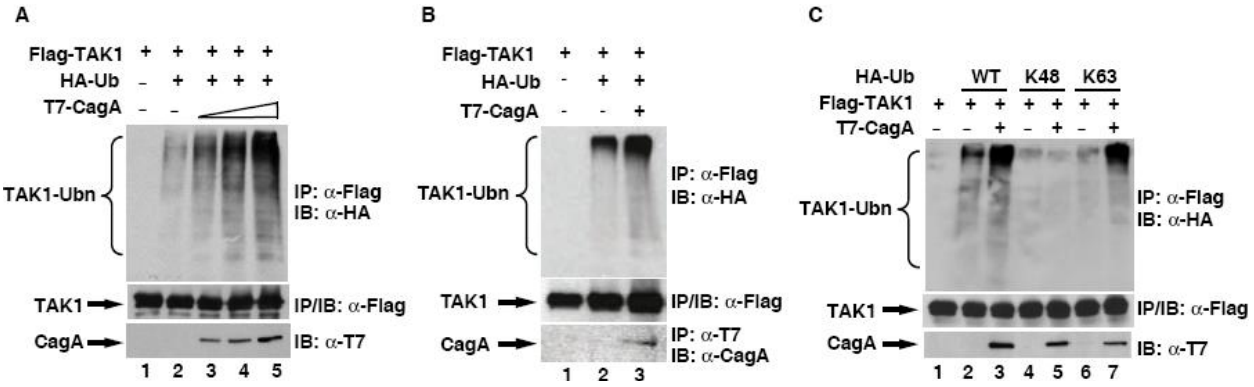

Figure 3.5. CagA enhances TAK1 ubiquitination. *A* Flag-TAK1 from HEK293T cells transfected as indicated was immunoprecipitated and immunoblotted for ubiquitination using HA antibodies. *B* Lysates from HEK293T cells transfected similarly to those in Fig. 3.5A were denatured before Flag-TAK1 was immunoprecipitated and immunoblotted for ubiquitination. *C* HEK293T cells were transfected with Flag-TAK1 with or without T7-CagA and with wt, Lys 48-only or Lys 63-only HA–ubiquitin as indicated. TAK1 ubiquitination was detected by immunoblotting Flag immunoprecipitates with HA antibodies.

other partner proteins of TAK1 are also known to be ubiquitinated, TAK1 itself is ubiquitinated in response to CagA (Fig. 3.5B). Further, the enhanced ubiquitination of TAK1 is Lys 63-linked rather than Lys 48-linked, as enhanced ubiquitination of TAK1 could only be detected in the presence of wild-type or Lys 63-only ubiquitin, but not in the presence of Lys 48-only ubiquitin (Fig. 3.5C), indicating that ubiquitination of TAK1 enhanced by CagA probably promotes the activity of TAK1 rather than its proteasomal degradation.

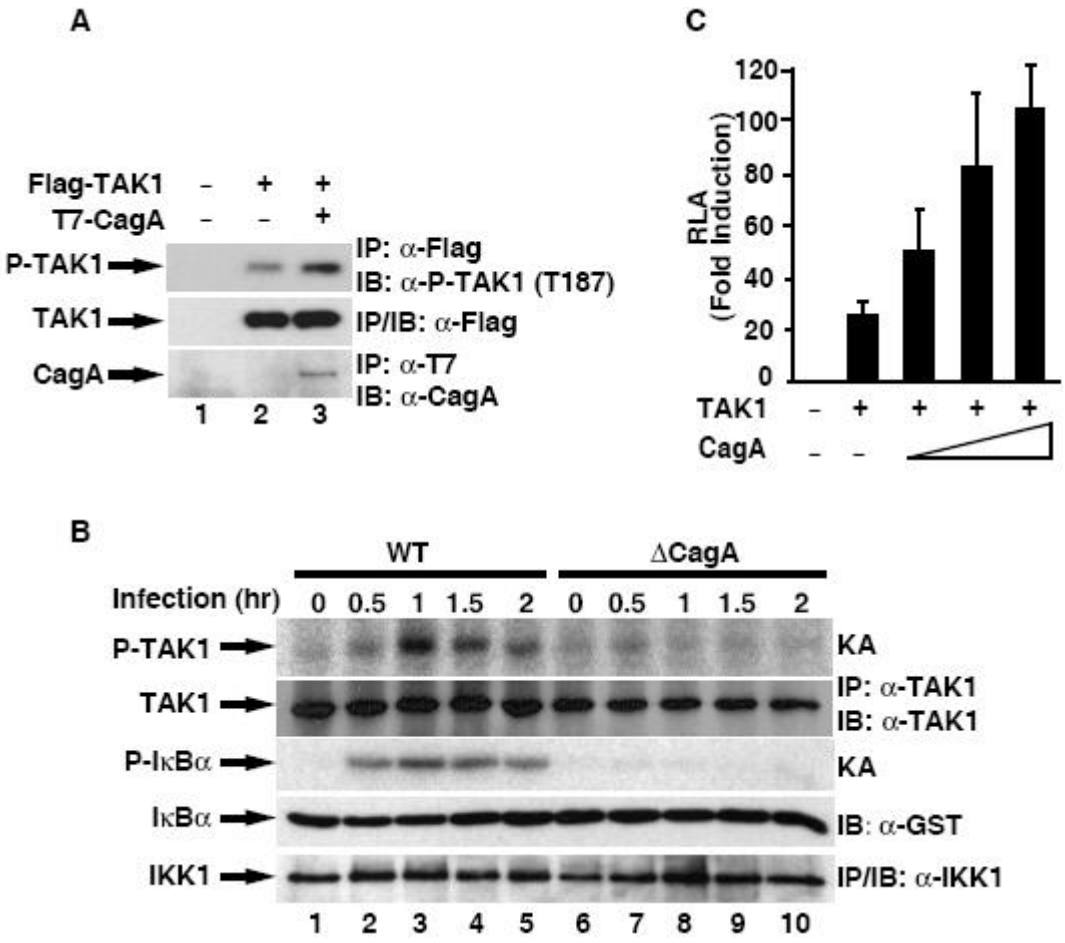

Figure 3.6. CagA enhances TAK1 activity. *A* 293T cells were transfected as indicated. Anti-Flag immunoprecipitates were separated by SDS-PAGE and immunoblotted for phosphorylated TAK1. *B* AGS cells were infected with wild-type or dCagA *H. pylori*; immunoprecipitates of TAK1 or IKK were assayed for kinase activity. *C* 293T cells were transfected with TAK1 and increasing amounts of CagA along with IL-8 promoter-driven luciferase. 36 hrs post-tranfection, luciferase activity was assayed and normalized to Renilla.

As CagA enhances the ubiquitination of TAK1 (Fig. 3.5), we next examined whether CagA could stimulate TAK1 kinase activity. One of the markers of TAK1 kinase activity is autophosphorylation of TAK1; threonines 178, 184, and 187 and serine 192 may be phosphorylated after activation by various stimulants (Singhirunnusorn et al., 2005; Yu et al., 2008). To evaluate kinase activation of TAK1 by CagA, CagA and TAK1 were co-expressed in 293T cells, and lysates were immunoblotted for phosphorylated TAK1 at T187; kinase activity

of TAK1 was enhanced by CagA (Fig. 3.6A). *H. pylori* infection in AGS cells also activated TAK1 and IKK with similar kinetics in a CagA-dependent manner, as observed in TAK1 and IKK kinase assays performed with immunoprecipitates from infected cells (Fig. 3.6B). Consistently, TAK1-mediated activation of NF-κB was enhanced by CagA in a dose-dependent manner in a κB-luciferase reporter assay (Fig. 3.6C).

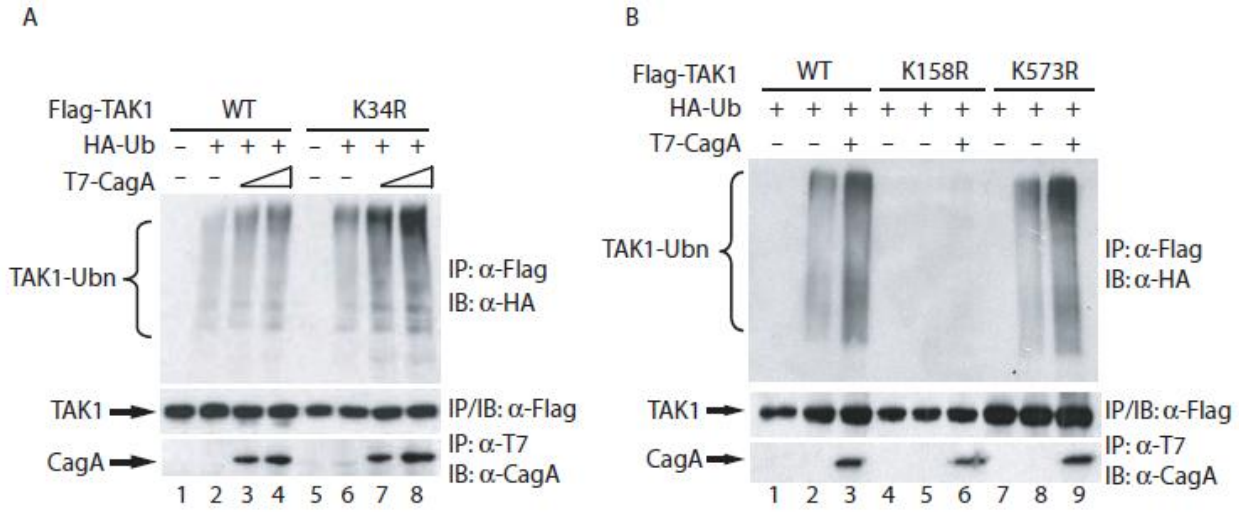

Figure 3.7. Ubiquitination of lysine 158 of TAK1 is enhanced by CagA. *A* & *B* Wild type and TAK1 lysine-to-arginine mutants were expressed in 293T cells along with ubiquitin and CagA. Anti-Flag precipitates were immunoblotted for HA.

CagA is localized to the plasma membrane and undergoes multimerization—two events that are essential for its signal transduction activity (Hatakeyama, 2008). Staining for overexpressed CagA and TAK1 showed that CagA colocalizes with TAK1 on the plasma membrane. While phosphorylation-deficient CagA induced ubiquitination to the same level as wild-type CagA, the multimerization-defective mutant of CagA failed to induce the ubiquitination of TAK1, a result which mirrors that observed in the mutants' abilities to activate

NF-κB (Fig. 3.6). It appears that the membrane-binding and oligomerization properties of CagA are likely important for the activation of TAK1 and NF-κB.

While it is apparent that CagA enhances the ubiquitination of TAK1, the importance of this ubiquitination is not completely clear. To better understand the role of ubiquitination of TAK1 in activation of the kinase and the NF-κB pathway, we looked for the specific lysine(s) being ubiquitinated in the presence of CagA. Various lysines have been reported as sites for ubiquitination in response to different stimuli, including TNF-α– and IL-1β-induced K158

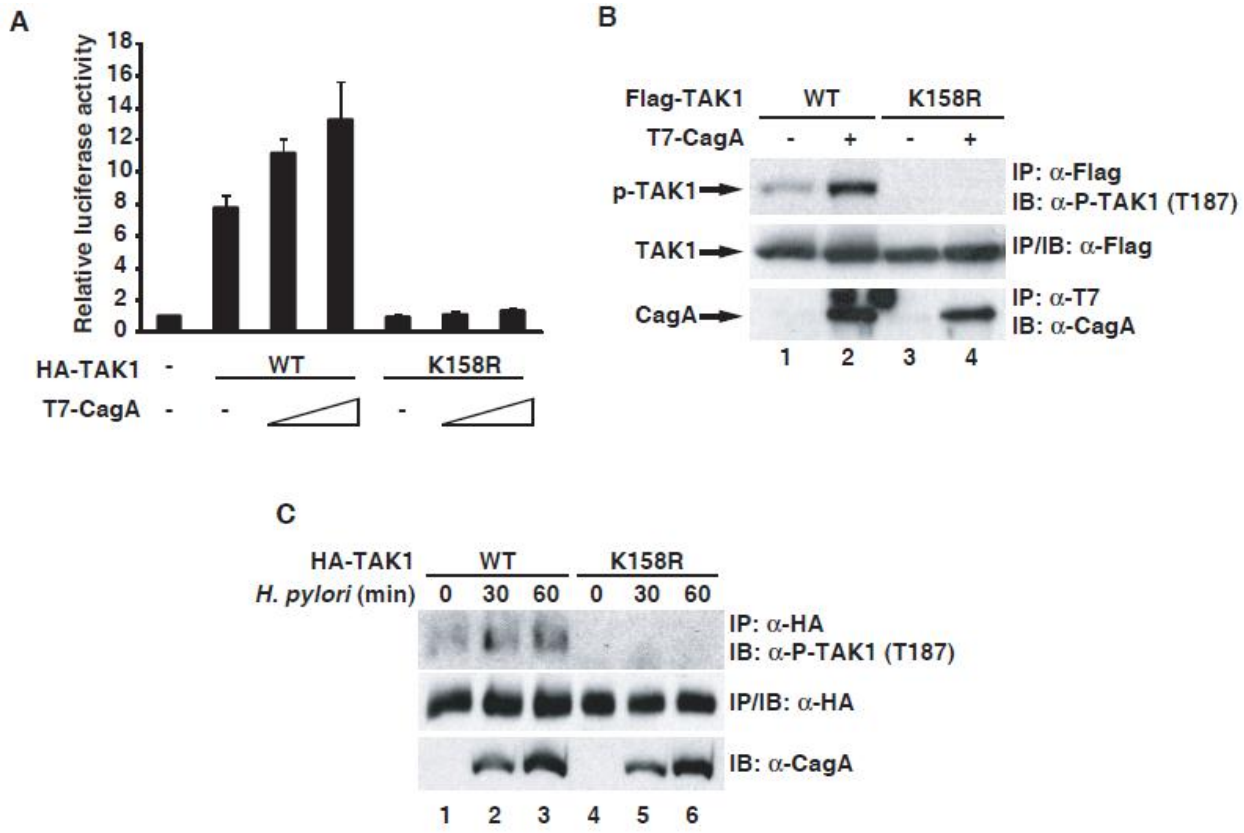

Figure 3.8. Ubiquitination at K158 of TAK1 is important for its activation by *H. pylori* CagA. ***A*** WT or K158R TAK1 were expressed in 293T cells along with IL-8-luciferase and Renilla with increasing amounts of CagA. Luciferase activity was assayed as in 3.6C. ***B*** WT or K158R TAK1 were expressed in 293T cells with or without CagA. Flag-TAK1 immunoprecipitates were immunoblotted for phosphorylated TAK1. ***C*** AGS cells expressing WT or K158R TAK1 were infected with *H. pylori* for indicated times. Flag-TAK1 immunoprecipitates were immunoblotted for phosphorylated TAK1.

ubiquitination and TGF-β-induced K34 ubiquitination (Fan et al., 2010; Sorrentino et al., 2008). Additionally, we found a potential site at K573 by mass spectrometry. To examine these sites, we created lysine-to-arginine mutants of each and overexpressed them in 293T cells along with ubiquitin and CagA. Mutation of K34 or K573 did not cause any reduction in either basal ubiquitination or in ubiquitination enhanced by CagA as compared to the wild-type (Fig 3.7). However, mutation of K158 resulted in considerably less basal ubiquitination, and no enhanced ubiquitination was observed with the addition of CagA. Ubiquitination of overexpressed K158R TAK1 after *H. pylori* infection in AGS cells was also significantly lower than that of overexpressed wild-type TAK1.

Having identified the lysine to which ubiquitin is added in response to *H. pylori* infection, we next went on to characterize the role which ubiquitination plays in *H. pylori*-induced TAK1 and NF-κB activation. In a luciferase reporter assay, IL-8-promoter luciferase was only induced by wild-type TAK1, and increased luciferase activity was only seen upon the addition of CagA to wild-type TAK1, not K158R mutant TAK1 (Fig. 3.8A).

Next, we looked at the kinase activity of TAK1. Autophosphorylation of wild-type TAK1 was increased when overexpressed along with CagA, but no basal or enhanced autophosphorylation of K158R TAK1 was observed (Fig. 3.8B). A similar result was seen when autophosphorylation of wild-type or K158R TAK1 was examined after *H. pylori* infection (Fig. 3.8C). All together, these data indicate that CagA enhances the ubiquitination of TAK1 at K158, a modification which is required for the *H. pylori*-induced activation of NF-κB.

3.4 Discussion

TAK1 is targeted by a number of different virulence factors from different pathogens, some to enhance and some to suppress NF-κB activity. Why TAK1 would be the target of choice for such a variety of pathogens is an interesting question, and one about which we may currently only hypothesize. TAK1 activates the IKK complex, a complex critical for the full activation of NF-κB. It may be that TAK1 is targeted by many microbial species because it plays such a crucial role in regulation of inflammation that circumventing the role of TAK1 is not an option for the host.

Finding that a key feature in the *H. pylori* activation of TAK1 is its ubiquitination at lysine 158 is a novel mechanism for pathogen interference in a host pathway. However, the role of K63-linked ubiquitination of TAK1 in its activation is not well understood. The E3 ligase just upstream of TAK1, TRAF6, ubiquitinates itself, and the zinc fingers in TAK1-binding proteins 2 and 3 (TAB2/3) specifically recognize the K63-linked chains and recruit the TAK1 complex to TRAF6 to be activated (Kanayama et al., 2004). Perhaps TAK1 ubiquitination is involved in a conformational change at that point, or perhaps TAK1 ubiquitination allows recruitment of other TAK1 complexes which in close proximity can promote cross-phosphorylation, resulting in active kinases. Other proteins in the pathway bind ubiquitin chains, such as NEMO, and ubiquitin binding by NEMO is required for activation of the IKK complex (Windheim et al., 2008).

A recent study challenges the idea that the ubiquitin chain must be anchored to a target protein to act. In their study, Xia et al find that free K63-linked ubiquitin chains, not attached to any other proteins, can bind the ubiquitin-binding domain of TAB2 and activate the TAK1 complex in vitro (Xia et al., 2009). Another study found that a TRAF6 mutant with no lysines

was competent at activating TAK1 and the NF-κB pathway in rescued TRAF6-deficient mouse embryonic fibroblasts, indicating that while the E3 ligase was required for activation of TAK1, ubiquitin chains attached to TRAF6 were not (Walsh et al., 2008). However, the free ubiquitin data was collected in vitro only, and would be difficult to determine in vivo. Also, two deubiquitinases, A20 and CYLD, have been found to be important for deubiquitinating targets such as RIP1 and TAK1 within the NF-κB pathway (Harhaj and Dixit, 2011). Such enzymes would seem irrelevant if ubiquitination of these proteins was not, in fact, required for some function within the pathway.

Lysine 158 in TAK1 appears to play a very important role in activation of the kinase and NF-κB. We found that ubiquitination of TAK1 when this lysine was mutated to arginine was sharply decreased, even when co-expressed with ubiquitin alone. The kinase activity and autophosphorylation of TAK1 K158R was also much less than the wild-type TAK1. Besides being a critical site of ubiquitination, the mutation of this lysine may also change the conformation of TAK1 in such a way as to make it a dead enzyme. Further studies, potentially involving protein crystallography, would be helpful to better define the role of this lysine.

CHAPTER 4

TAK1 UBIQUITINATION IN RESPONSE TO *HELICOBACTER PYLORI* REQUIRES E2 UBC13 AND E3 TRAF6

4.1 Introduction

Like many other modifications, ubiquitination is a modification which required enzymes to specifically recognize and attach a molecule to a target protein. In the process, illustrated in Figure 4.1, one of the two known E1 enzymes uses ATP to form a thioester bond between a

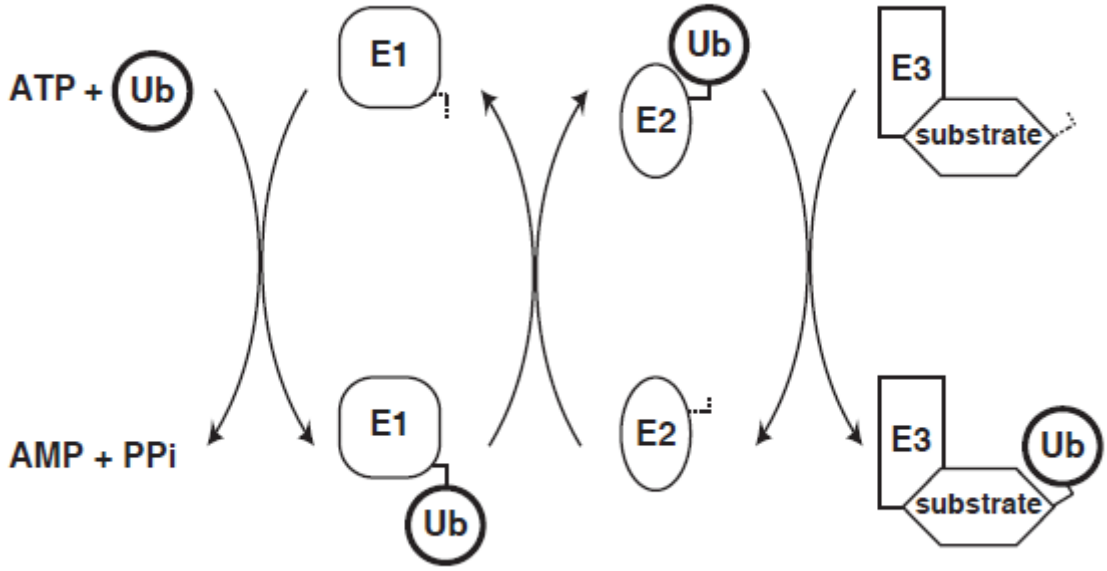

Figure 4.1. Schematic of ubiquitination catalysis.

cysteine within the E1 and the C-terminus of ubiquitin, a small, 76-amino acid protein. Next, ubiquitin is transferred to an ubiquitin-conjugating enzyme, E2, which also binds ubiquitin through a thioester bond. Finally, an E3 ligase specifically guides the ubiquitin to covalently attach to a target protein via an isopeptide bond between the carboxy terminus of ubiquitin and ε-amino group of a lysine on the target. A single ubiquitin molecule may be added to a target

protein, or a chain of ubiquitins may be added, attached processively from one of seven internal lysines within ubiquitin to the C-terminus of the next ubiquitin. More than forty E2s and hundreds of E3s are found in mammalian genomes. Ubiquitin conjugating E2s are thought to be responsible for the types of ubiquitin-ubiquitin linkages which are constructed in ubiquitin chains, and E3 ligases direct the ubiquitination of specifically recognized targets.

To better understand the pathway utilized for *H. pylori*-induced TAK1 ubiquitination and activation, we sought to determine the ubiquitin conjugating E2 and the E3 ligase required for TAK1 ubiquitination, and the requirement for each in the activation of NF-κB by *H. pylori* infection.

4.2 Materials and Methods

Cell lines, *H. pylori* culture and infection. Human AGS gastric adenocarcinoma and HEK293T cells were cultured in DMEM supplemented with 10% fetal bovine serum. *H. pylori* G27 was cultured in bisulphite-free *Brucella* broth on agar media containing Ham's F-12 medium supplemented with 10% fetal bovine serum and 5 μg/ml vancomycin at 37°C in the presence of 10% CO_2. *H. pylori* was added to AGS cells for infection at a multiplicity of infection of 50–100.

Plasmids. CagA was amplified by PCR from the genomic DNA of *H. pylori* strain G27 and subcloned into the pcDNA3.1-Flag or pcDNA3.1-T7 vector. TRAF6 was subcloned into the pcDNA3.1-T7 vector. HA-TAK1 was received as a gift from Dr. J. Ninomiya-Tsuji and Flag-TAK1 was received as a gift from Dr. X. Lin. His-MKK6 (K82A) was received as a gift from Dr. Z. J. Chen.

Antibodies. Normal rabbit IgG, antibodies against CagA, TAK1, TRAF6, Flag, HA, His and ubiquitin were from Santa Cruz Biotechnology. Anti-T7 antibody was from Covance. Phospho-S536 RelA, phospho-IκBα (S32/36) and phospho-TAK1 (T184/187) were from Cell Signaling.

siRNA knock down. Ubc13, Uev1a, and UbcH7 siRNA from Ambion (Ubc13-A sense: CCAGAUGAUCCAUUAGCAAtt; Ubc13-B sense: GCAUGGACUAGGCUAUAUUtt; Uev1a sense: ACUUACAAGAUGGACAGGGtt; UbcH7 sense: AUGUGGGAUGAAAAACUUCtt) was transfected into AGS cells using Lipofectamine 2000 according to the manufacturer's protocol. 24 hours post-transfection, cells were transfected with Flag-TAK1, HA-TAK1, HA-Ub, and/or T7-CagA as described, and 24 hours later cells were infected or harvested for further experiments.

Immunoprecipitation, immunoblotting analysis and EMSA. Lysates were immunoprecipitated for 2 hours at 4°C using 20 μL of a slurry containing 50% anti-T7-conjugated or anti-Flag-conjugated agarose beads (Novagen or Sigma), or with 5 μL of various antibodies and 20 μL Protein A agarose beads (Sigma). These immunoprecipitates were washed three times in lysis buffer. Lysates from HEK293T or AGS cells were collected, cleared and denatured in SDS sample buffer. Lysates or immunoprecipitates were separated by SDS-PAGE (10-13%), transferred to nitrocellulose transfer membranes and immunoblotted with various antibodies followed by visualization of the immunoreactive proteins by ECL (Amersham).

Quantitative real-time PCR analysis. AGS cells were infected with *H. pylori* for various times and total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Complementary DNA was synthesized using the Omniscript RT kit (Qiagen). Quantitative real-time PCR was performed using the Qiagen SYBR green PCR kit, with the aid of the 7300 Real-

time PCR system (ABI, Foster City, CA, USA). PCR primers for human β-actin, IL-8 and TNF-α were purchased from Qiagen.

Transient transfection and luciferase reporter assay. For experiments using HEK293T cells, transfection was performed using the calcium phosphate method. For experiments using AGS cells, either calcium phosphate or Lipofectamine 2000 was used to transfect cells (Invitrogen). For luciferase assay, cells were lysed in passive lysis buffer 30 hours post-transfection; firefly and renilla luciferase activities were measured with the dual luciferase assay system from Promega. Firefly luciferase activity was normalized to renilla luciferase activity. Results represent the average of three independent experiments ± s.d.

Establishment of cell lines stably expressing TRAF6 shRNA. The TRAF6-specific short hairpin RNA oligomer (5'-GGAGAAACCTGTTGTGATT-3') was subcloned into the pSUPER-retro-neo vector (Oligoengine). The vector was transfected into Phoenix-Ampho packaging cells and the supernatants were collected 48 h after transfection and used to infect AGS cells in the presence of 10 μg/ml polybrene (Sigma) for 24 h. After changing the medium, cells were cultured for 16 h and then selected with 0.5 mg/ml G418 (Sigma) for 7 days. The knock-down efficiency was assessed by immunoblotting.

4.3 Results

We first sought to determine the ubiquitin-conjugating enzyme required for TAK1 ubiquitination. Only a few E2s have been found to play roles in catalyzing K63-linked ubiquitination, including Ubc13 which pairs with either Uev1a or Mms2, and potentially UbcH7 (David et al., 2010; Geetha et al., 2005). An ubiquitination assay for TAK1 was performed in 293Ts overexpressing TAK1, ubiquitin, and CagA after Ubc13, Uev1a and UbcH7 were first

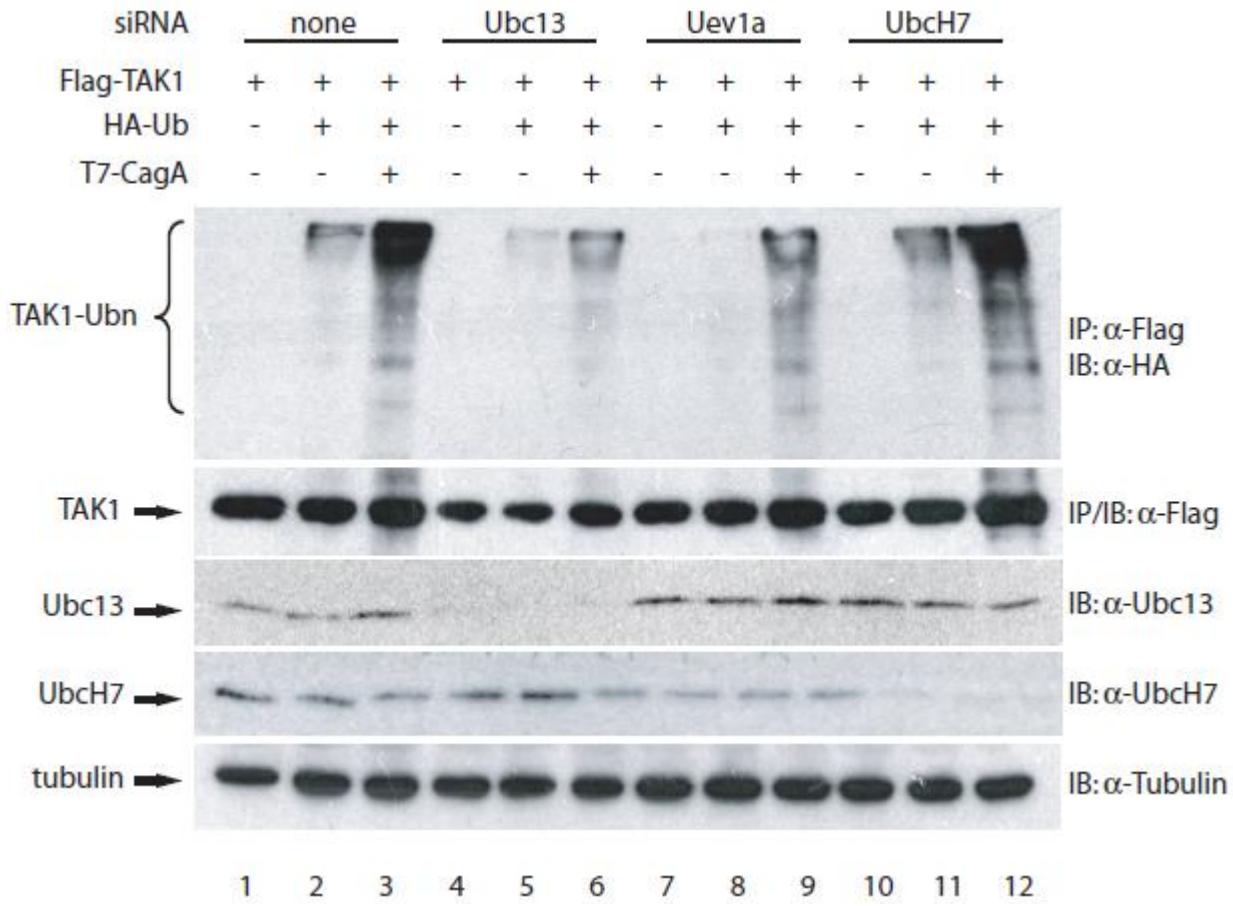

Figure 4.2. Ubc13 and Uev1a likely work together as the E2 ubiquitin conjugating enzyme for TAK1. siRNA against different E2 enzymes were transfected into 293T cells, then TAK1 was overexpressed along with HA-ubiquitin and CagA. Anti-Flag immunoprecipitates were immunoblotted for HA.

knocked down with siRNA. Reduced ubiquitination of TAK1 was seen when Ubc13 and Uev1a were knocked down, suggesting that these E2s are required for the ubiquitination of TAK1 (Fig. 4.2). Because Ubc13 and Uev1a typically work together, with neither capable of conjugating ubiquitin alone (David et al., 2010), further studies were performed investigating Ubc13 alone. Knock down of Ubc13 in AGS cells reduced the ubiquitination of TAK1 induced by *H. pylori* infection, further confirming the role which Ubc13 plays in *H. pylori*-induced TAK1 ubiquitination (Fig. 4.3).

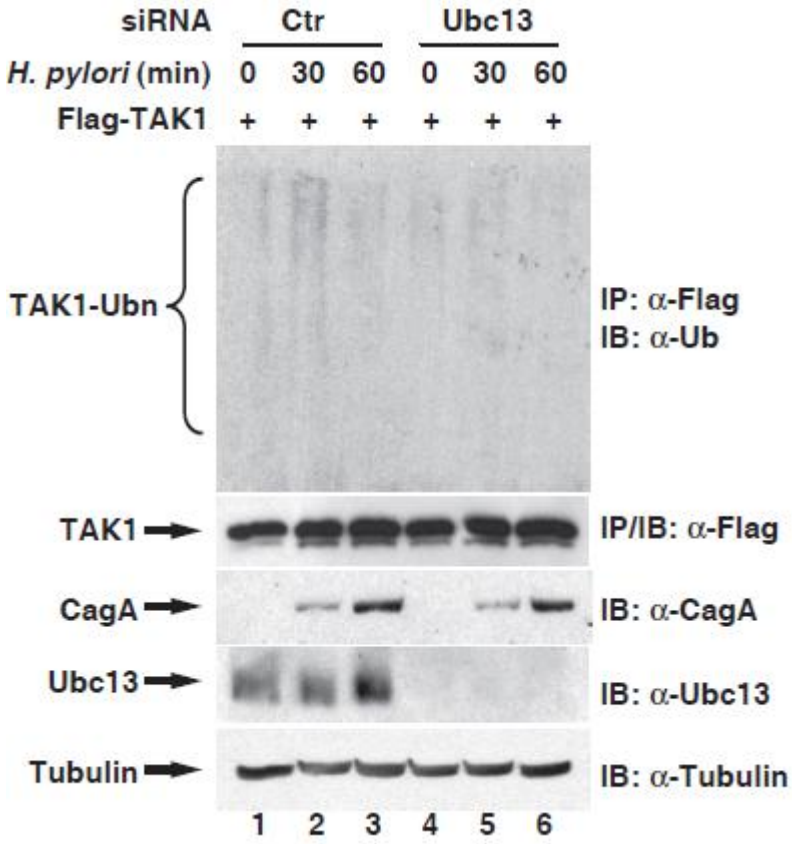

Figure 4.3. Ubc13 is required for *H. pylori*-induced ubiquitination of TAK1. AGS cells expressing Flag-TAK1 were infected as indicated with *H. pylori*. Flag immunoprecipitates were immunoblotted for ubiquitin.

After determining that Ubc13 is required for the ubiquitination of TAK1 in response to *H. pylori* infection, we were interested to see whether knock down of the E2 diminished the CagA-mediated and the *H. pylori*-induced activation of TAK1 and NF-κB. First, 293T cells were transfected with TAK1, CagA, and increasing amounts of a dominant negative form of Ubc13, Ubc13C87A (Deng et al., 2000). Luciferase activity was measured 30 hours post-transfection. When the dominant negative form of Ubc13 was co-transfected, TAK1-stimulated NF-κB activity and CagA-enhanced TAK1-NF-κB activity were both decreased in a dose-dependent fashion (Fig. 4.4A). Next, CagA-induced TAK1 autophosphorylation was investigated with and

without Ubc13C87A. Co-transfection of the dominant-negative Ubc13 severely diminished CagA-enhanced TAK1 phosphorylation (Fig. 4.4B). Lastly, Ubc13 was knocked down in AGS cells, cells were infected with *H. pylori*, and HA-TAK1 was immunoprecipitated from the cells.

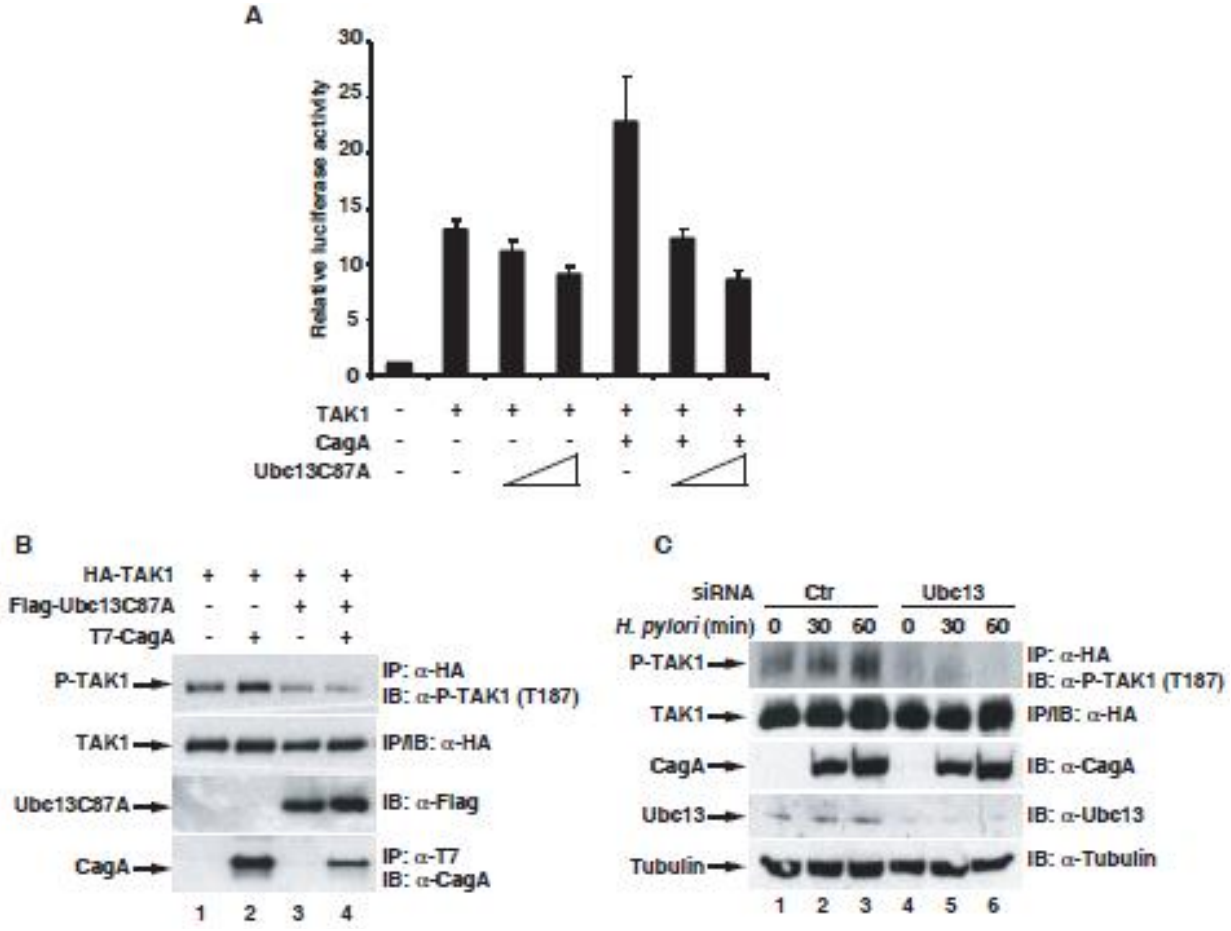

Figure 4.4. Ubc13 is required for CagA-dependent *H. pylori*-induced TAK1 and NF-κB activity. ***A*** IL-8-luciferase, Renilla, and TAK1 were expressed in 293T cells with and without CagA along with increasing amounts of Ubc13C87A. Luciferase activity was assayed as in Fig. 3.6C. ***B*** TAK1 and CagA were expressed in 293T cells with or without Ubc13C87A. HA-TAK1 immunoprecipitates were immunoblotted for phosphorylated TAK1. ***C*** AGS cells expressing TAK1 and either control or Ubc13 siRNA were infected with *H. pylori* as indicated. HA-TAK1 immunoprecipitates were immunoblotted for phosphorylated TAK1.

Autophosphorylation of TAK1 was detected using α-TAK1 T187 antibodies. In cells with Ubc13 knocked down there was less autophosphorylation of TAK1 and less TAK1 kinase

activity in response to *H. pylori* infection than in cells where a control siRNA was transfected (Fig 4.4C).

NF-κB activation in Ubc13 knock-down cells was also investigated. When Ubc13 was

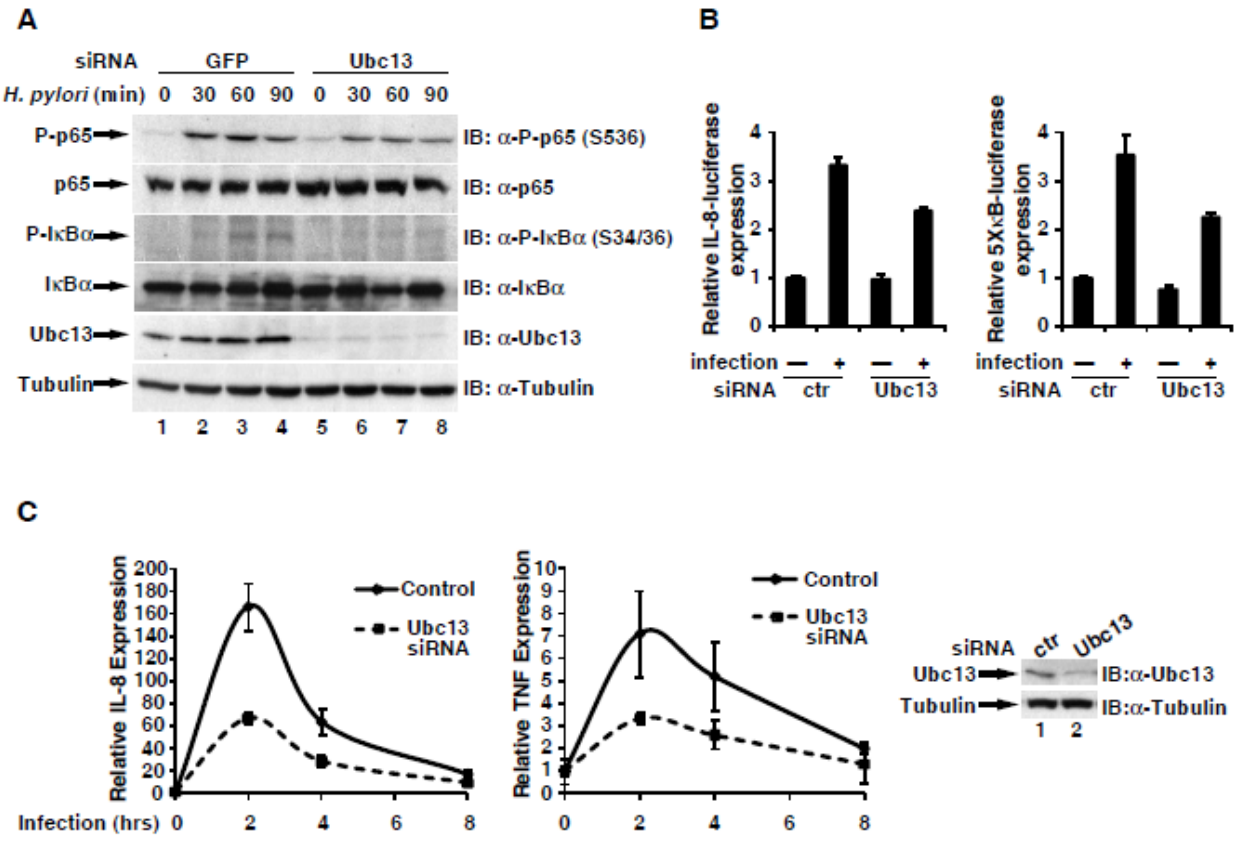

Figure 4.5. Ubc13 is important for *H. pylori*-induced NF-κB activation. AGS cells were transfected with control or Ubc13 siRNA, then infected with *H. pylori* as indicated. ***A*** Lysates were immunoblotted as stated. ***B*** Cells were infected with *H. pylori* for 6 hrs, then lysates were assayed for luciferase activity. ***C*** mRNA was collected from cells and RT-PCR was performed to determine NF-κB gene transcription.

knocked down and cells were infected with *H. pylori*, p65 and IκBα phosphorylation was reduced as detected by Western blotting of cell lysates, indicating a reduced capacity to activate NF-κB (Fig. 4.5A). When Ubc13 was knocked down and cells were infected with *H. pylori*, luciferase assays and RT-PCR showed significantly less NF-κB transcription activity than cells

transfected with a siRNA control (Fig. 4.5B & C). All together these data show that Ubc13 is not only required for TAK1 ubiquitination, but is also required for the activation of NF-κB by *H. pylori* infection.

Others have reported that the ubiquitination of TAK1 effected by TGF-β or TNF-α

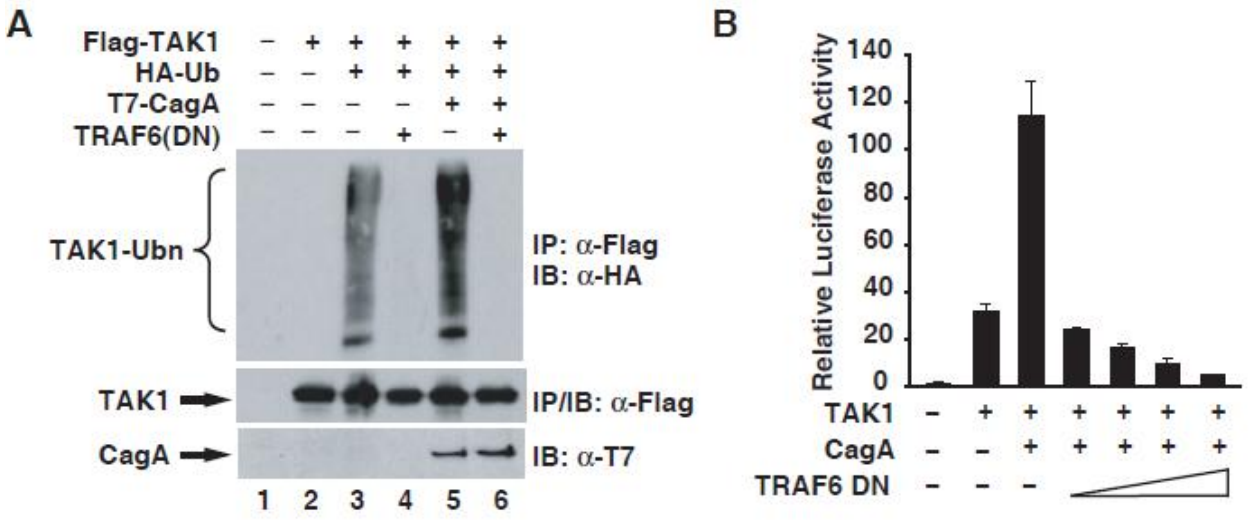

Figure 4.6. Dominant-negative TRAF6 blocks CagA-enhanced TAK1 ubiquitination and activation of NF-κB. ***A*** 293T cells were transfected as indicated. Flag-TAK1 immunoprecipitates were immunoblotted for HA-ubiquitin. ***B*** 293T cells were transfected for IL-8-luciferase assay as indicated. 30hrs post-transfection, lysates were assayed for luciferase assay.

stimulation requires E3 ligase TRAF6, an E3 ligase whose activity first hinted at a role for ubiquitination in the activation of TAK1 (Wang et al., 2001). To determine whether TRAF6 was the E3 responsible for CagA-induced ubiquitination of TAK1, we first assessed the effect of a dominant-negative mutant of TRAF6 (TRAF6-DN; (Jono et al., 2004) on the polyubiquitination of TAK1. TRAF6-DN not only blocked the ubiquitination of TAK1, but also inhibited the enhanced ubiquitination of TAK1 facilitated by CagA (Fig 4.6A). The ability of CagA to enhance TAK1-mediated NF-κB activation was also inhibited by TRAF6-DN (Fig 4.6B), indicating that ubiquitination of TAK1 is crucial for its activation.

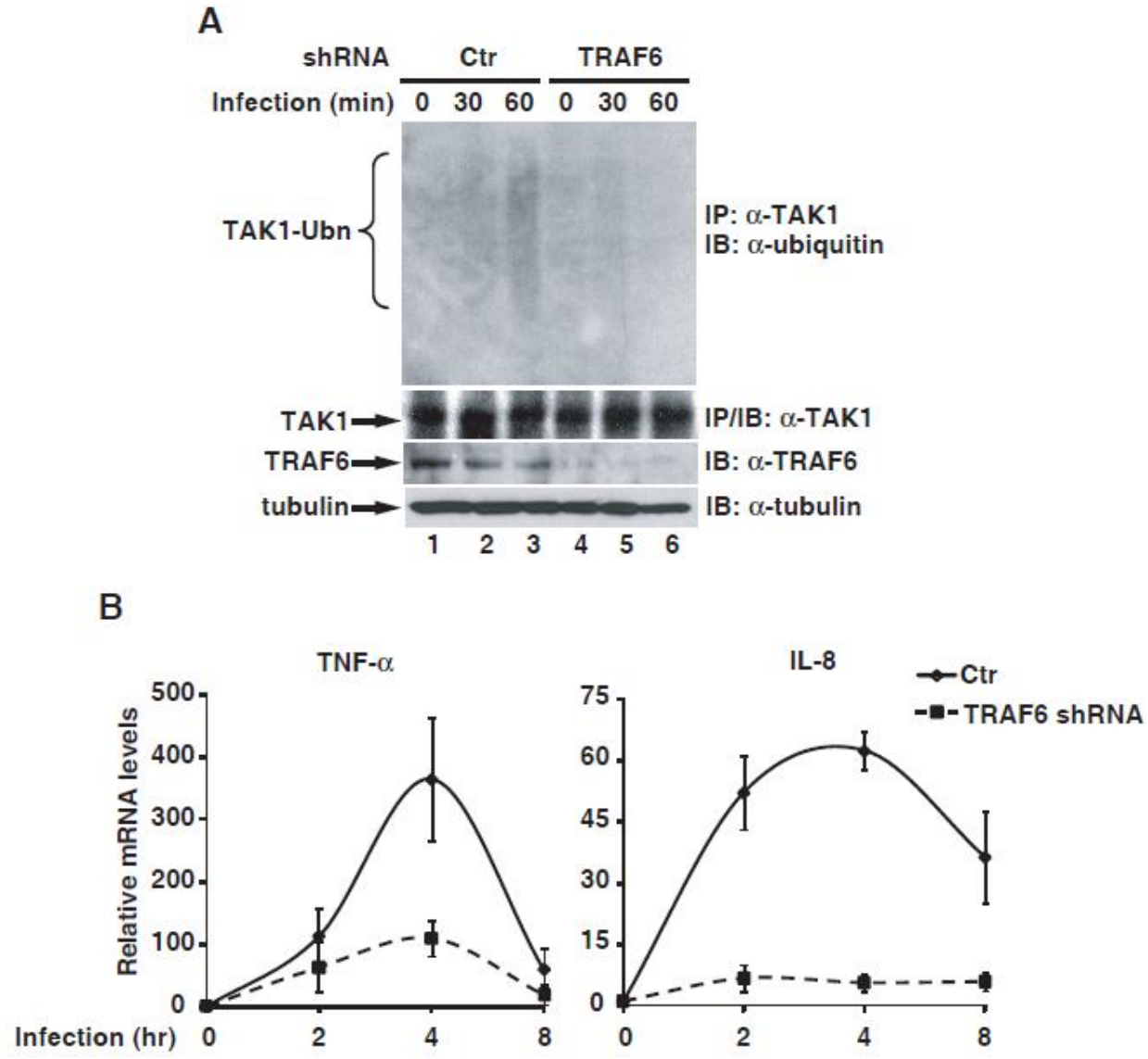

Figure 4.7. TRAF6 is required for *H. pylori*-induced TAK1 ubiquitination and NF-κB activation. AGS stably transfected with TRAF6 shRNA were infected with *H. pylori* as indicated. ***A*** TAK1 immunoprecipitates were immunoblotted for ubiquitin. ***B*** RT-PCR was performed to measure NF-κB target gene transcription.

To analyze further the role of TRAF6 in *H. pylori*-induced ubiquitination of TAK1 and NF-κB activation, we generated an AGS cell line stably expressing short hairpin RNA (shRNA) against TRAF6 and examined the ubiquitination of TAK1 in these cells. *H. pylori*-induced

ubiquitination of TAK1 and mRNA expression of IL-8 and TNF-α were markedly attenuated in the TRAF6-depleted cells compared with AGS cells stably expressing control shRNA (Fig 4.7A & B). These data demonstrate that ubiquitination of TAK1 is crucial for CagA-enhanced NF-κB activation and that TRAF6 is likely the E3 ubiquitin ligase for *H. pylori*-induced TAK1 ubiquitination.

4.4 Discussion

Around forty E2 ubiquitin conjugating enzymes have been found within the human genome. E2s are easily distinguished by the Ubc domains found in their protein sequences. Many participate in the synthesis of lysine 48-linked ubiquitin chains to target proteins for degradation by the proteasome. Only a few have been found to play a role in synthesis of other types of ubiquitin polymers, including lysine 63-linked, lysine 11-linked, and linear ubiquitin chains. Studies suggest that linkage specificity may be determined either by an E2 alone or by E2-E3 combinations in vivo (Ye and Rape, 2009). The orientation of the next ubiquitin molecule to be added towards the last-added ubiquitin molecule is believed to be the determining factor in chain linkage specificity, and structural evidence continues to support this model.

Ubc13 is one of only a handful of E2s capable of catalyzing K63-linked ubiquitin chains, and one of the most well-studied. In addition to activation of the NF-κB signaling pathway, K63-linked ubiquitination is also involved in DNA damage response pathways and regulation of lysosomal degradation processes (David et al., 2010). This wide variety of functions makes modulation of K63-linked ubiquitination a potentially useful target for anti-cancer or immune-regulatory therapeutics. While K63-linked ubiquitination is a relatively newly discovered

modification, future studies may show that its regulation may be as important a target for new therapies as phosphorylation or acetylation are currently.

Although CagA enhances TRAF6-mediated Lys 63-linked ubiquitination of TAK1, it is not clear how this occurs. CagA fails to enhance the interaction between TRAF6 and TAK1 (Appendix Fig. A.1), excluding the possibility that CagA might function as a bridging factor to facilitate the TAK1–TRAF6 interaction. It is possible that CagA could target TRAF6 and enhance its E3 ligase activity. Supporting this argument, we found that CagA also interacts with TRAF6 *in vitro* and enhances its auto-ubiquitination (Appendix Fig. A.2 & A.3). CagA is associated with the membrane when it enters the host cell, and has been shown to oligomerize (Higashi et al., 2005; Ren et al., 2006). It is possible that this oligomerization recruits TRAF6 which enhances its E3 ligase activity and thus the ubiquitination of TAK1. Further supporting this, our data shows that oligomerization-deficient CagA does not activate NF-κB (Fig. 2.5). Another possibility is that CagA may inhibit deubiquitination by A20 or CYLD, two de-ubiquitinases (DUBs) shown to remove ubiquitin chains from TRAF6 and TAK1 (Boone et al., 2004; Kovalenko et al., 2003; Reiley et al., 2007), thereby enhancing the overall ubiquitination of TAK1. CagA may physically interfere with these DUBs, or may affect their NF-κB-regulated expression. Future studies should examine these possibilities.

How the ubiquitination of TAK1 activates the kinase is also unclear. It is expected that, like other K63-linked ubiquitinated signaling molecules, the polyubiquitin chain functions as a scaffold to recruit other signaling molecules which are essential for the activation of TAK1. For example, MEKK3 has been shown to be recruited to the ubiquitinated TAK1, and this recruitment is required for the activation of TAK1 (Yamazaki et al., 2009). Also, TAK1 ubiquitination may aid in its activity by recruiting substrates, such as the IKK complex. The

ubiquitin-binding domain of the regulatory subunit NEMO has been shown to be important in the activation of IKK (Adhikari et al., 2007), and it may be that it binds to the ubiquitin chains on TAK1. TAK1 binding proteins TAB2 and TAB3 contain novel zinc fingers which bind specifically to K63-linked ubiquitin chains (Kanayama et al., 2004). Ubiquitination of TAK1 may allow for the recruitment of other TAK1-TAB2/3 complexes, and the interaction of multiple complexes allows for cross-activation of TAK1. Another possibility is that TAK1 ubiquitination simply changes the conformation of the kinase, allowing for its activation.

CHAPTER 5

POST-TRANSLATIONAL MODIFICATIONS OF NF-κB ARE IMPORTANT FOR *HELICOBACTER PYLORI*-INDUCED INFLAMMATION

5.1 Introduction

NF-κB is a tightly controlled transcription factor, with many levels of regulation. While we have mainly focused on the upstream pathways which lead to NF-κB activity, its regulation once it reaches the nucleus is just as important. Many factors have been discovered to contribute to the transcriptional activation of NF-κB target genes, including the binding of different homo- or heterodimers of NF-κB to the cognate κB sites, the recruitment of various basal transcriptional factors and coactivators to the promoters, and the modifications of the histone tails around the promoters of NF-κB target genes (Chen and Greene, 2004). Recent studies indicate that posttranslational modifications of NF-κB, especially of the RelA subunit, play a critical role in fine-tuning the transcriptional activity of NF-κB, adding another important layer of complexity to the transcriptional regulation of NF-κB.

A role for phosphorylation of RelA in the regulation of NF-κB activity has long been suggested (Baeuerle and Baltimore, 1996). Accordingly, many kinases and phosphorylation sites including, S276, S311, and S536, have been identified. RelA can be phosphorylated both in the cytoplasm and in the nucleus in response to a variety of stimuli. Most of the phosphorylation sites are within the N-terminal RHD and the C- terminal transcriptional activation domains. Phosphorylation of these sites often results in increased levels of transcription, though decreased transcription may also result, depending on the sites of phosphorylation, the target genes, and the stimuli.

Acetylation is another important posttranslational modification of RelA that has been extensively studied over the years. Different from phosphorylation, acetylation mostly occurs in the nucleus, where most of the enzymes mediating this modification reside. These enzymes include histone acetyltransferases and histone deacetylases, which mediate the addition or removal of the acetyl group to and from lysine residues. Reversible acetylation of RelA regulates diverse functions of NF-κB, including DNA binding activity, transcriptional activity, and its ability to associate with IκBα, and plays important roles in the NF-κB-mediated inflammatory response and cancer. Seven acetylated lysines have been identified within RelA, including lysines 122, 123, 218, 221, 310, 314 and 315. The majority of these lysines are acetylated by p300/CBP, but some lysines, like 122 and 123, can be acetylated by PCAF as well (Kiernan et al., 2003). Acetylation of these different lysines modulates distinct functions of NF-κB.

Finally, lysine methylation has recently emerged as another important modification for the regulation of nuclear NF-κB function. The functional consequence of methylation depends on both position and state of the methylation site, since lysine can be mono-, di-, or tri-methylated. Several methyltransferases have been identified to methylate RelA and regulate distinct functions of NF-κB. For example, SET7/9 specifically monomethylates RelA at K314 and K315 in vitro and in vivo (Yang et al., 2009b). TNF-α- or LPS-induced methylation of RelA by Set9 negatively regulates the function of NF-κB by inducing the ubiquitination and degradation of promoter-bound RelA, which represents a novel mechanism by which NF-κB transactivation can be turned off.

Although many of the above mentioned studies using biochemical and molecular approaches have demonstrated that various posttranslational modifications of RelA regulate distinct biological functions, especially the transcriptional activation of NF-κB, the physiological

significance of these modifications is still being investigated. Nevertheless, a growing number of studies have linked various posttranslational modifications to a variety of disease conditions, especially inflammatory diseases and cancer. For example, phosphorylation of RelA on S276 or S536 is important for polymicrobial infection-induced lung inflammation (Kweon et al., 2006). Viruses, such as respiratory syncytial virus, also stimulate the phosphorylation of RelA (Jamaluddin et al., 2009). Anti-inflammatory drugs, such as licochalcone A, which act by inhibiting phosphorylation of RelA, may prove this modification to be an important target (Furusawa et al., 2009). Additionally, activation of NF-κB by different pathogens is often associated with hyperacetylation of NF-κB. *H. influenzae* (NTHi) induces the acetylation of RelA, and this acetylation is essential for NTHi-induced NF-κB activation and inflammation (Ishinaga et al., 2007; Ishinaga et al., 2009). Pathogens including *Mycobacterium tuberculosis, M. leprae, Candida albicans*, measles virus, and human immunodeficiency virus-1 (HIV), trigger DC-SIGN and induce acetylation of RelA, resulting in both prolonged and increased IL-10 transcription to enhance the anti-inflammatory cytokine response (Gringhuis et al., 2007). Hyperacetylated RelA has been found in many cancer cells, and acetylation might account for the constitutively active NF-κB in cancer cells (Dai et al., 2005). A compound shown to mediate antitumor and anti-inflammatory activities, anacardic acid, has been found to inhibit NF-κB acetylation by p300 histone acetyltransferase (Sung et al., 2008). Clearly, modulating NF-κB activity at the level of posttranslational modifications could prove a promising new field for cancer and anti-inflammatory therapeutics.

NF-κB posttranslational modifications are induced by numerous pathogens resulting in chronic activation of the transcription factor. Thus, we were interested to determine whether *H. pylori* infection might also affect posttranslational modifications of NF-κB. If so, therapeutics

targeted against these modifications might prove useful to down regulate *H. pylori*-induced chronic inflammation and cancer.

5.2 Materials and Methods

Cell lines, *H. pylori* culture and infection. Human AGS gastric adenocarcinoma and MEF cells were cultured in DMEM supplemented with 10% fetal bovine serum. *H. pylori* G27 was cultured in bisulphite-free *Brucella* broth on agar media containing Ham's F-12 medium supplemented with 10% fetal bovine serum and 5 μg/ml vancomycin at 37°C in the presence of 10% CO_2. *H. pylori* was added to AGS cells for infection at a multiplicity of infection of 50–100.

Quantitative real-time PCR analysis. AGS cells were infected with *H. pylori* for various times and total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Complementary DNA was synthesized using the Omniscript RT kit (Qiagen). Quantitative real-time PCR was performed using the Qiagen SYBR green PCR kit, with the aid of the 7300 Real-time PCR system (ABI, Foster City, CA, USA). PCR primers for human β-actin, IL-8 and TNF-α were purchased from Qiagen.

siRNA knock down and luciferase assay. Brd4 siRNA from Ambion was transfected into AGS cells using Lipofectamine 2000 according to the manufacturer's protocol. For luciferase reporter assays, calcium phosphate was used to transfect cells. Cells were lysed in passive lysis buffer 30 hours post-transfection; firefly and renilla luciferase activities were measured with the dual luciferase assay system from Promega. Firefly luciferase activity was normalized to renilla luciferase activity. Results represent the average of three independent experiments ± s.d.

5.3 Results

In previous chapters we have demonstrated that *H. pylori* CagA activates the phosphorylation of p65 at serine 536 (Fig. 2.6). An interplay exists between phosphorylation at S536 and acetylation of RelA, in that phosphorylation enhances the binding of the histone acetyltransferase p300, providing enhanced acetylation of lysine 310, another modification for the positive regulation of NF-κB activity (Huang et al., 2010)(Huang et al, 2010). We therefore decided to explore whether acetylation of RelA plays a role in *H. pylori* activation of NF-κB. First, AGS cells treated with trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, were infected with *H. pylori*. NF-κB-driven luciferase activity was measured, and inhibition of HDACs sensitive to TSA increased the *H. pylori*-induced activation of NF-κB (Fig. 5.1).

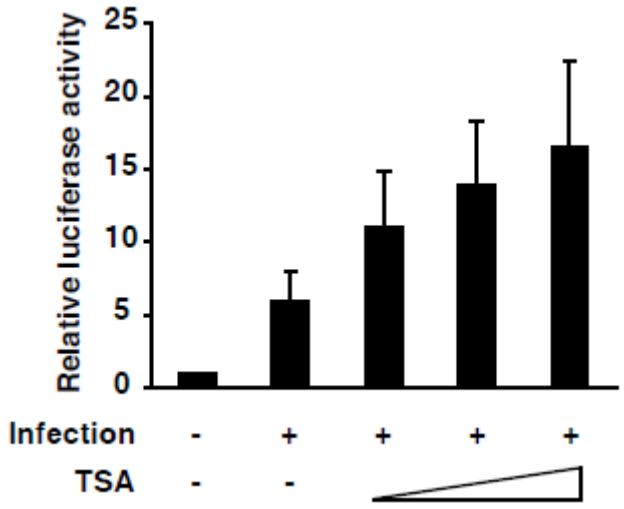

Figure 5.1. Histone deacetylase inhibitor TSA enhances NF-κB activation by *H. pylori*. AGS cells were transfected with IL-8-luciferase and renilla. 30 hrs post-transfection cells were treated with increasing doses of TSA and infected with *H. pylori* for 8 hours. Luciferase activity was measured and normalized to renilla.

Recently, acetylation of p65 at K310 was found to upregulate NF-κB gene transactivation by recruiting a co-factor, Brd4, which recruits and activates the RNA polymerase II kinase CDK9, a crucial step in transcription activation (Huang et al., 2009). We therefore sought to determine whether Brd4 recruitment to K310 might play a role in *H. pylori* transactivation of NF-κB. Brd4 was knocked down in AGS cells using siRNA, then cells were infected with *H. pylori* and NF-κB target gene mRNA was measured with RT-PCR. NF-κB activation by *H. pylori* was significantly decreased when Brd4 was knocked down as compared with a control (Fig. 5.2).

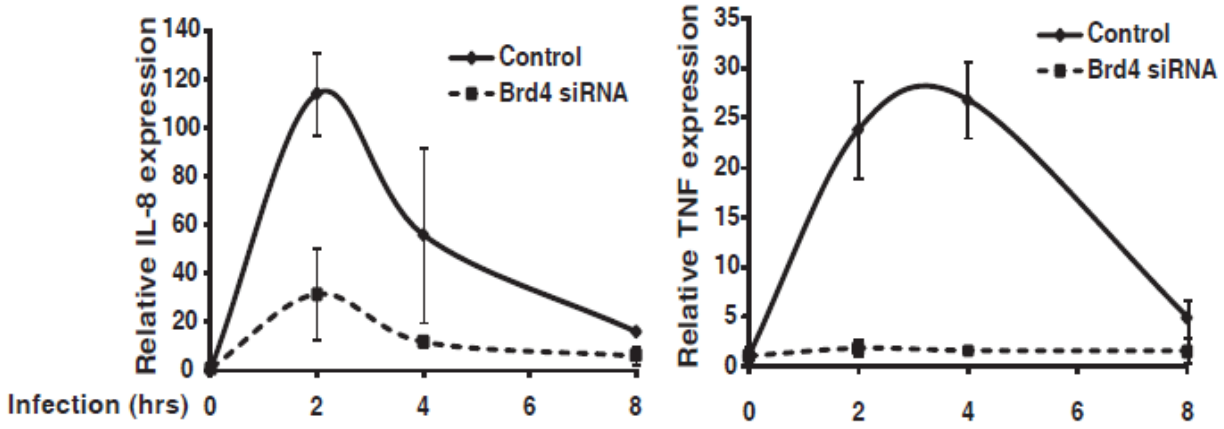

Figure 5.2. Brd4 is required for *H. pylori*-induced NF-κB activation. AGS cells were transfected with Brd4 siRNA or control siRNA. Cells were infected with *H. pylori* for indicated times, and mRNA was harvested for RT-PCR.

Brd4 binds to acetylated p65 through its two bromodomains, domains recognized for their ability to specifically recognize acetylated lysine, and the interaction is required for Brd4 to recruit CDK9 to the promoter-bound NF-κB transcriptional complex (Huang et al., 2009). To explore whether this specific interaction is required for *H. pylori*-induced NF-κB activity, we treated cells with a newly-identified cell-permeable small molecule inhibitor of bromodomain-containing factors, JQ1 (Filippakopoulos et al., 2010). JQ1 and DMSO treated AGS cells were

infected with *H. pylori*, and NF-κB target gene mRNA was detected using RT-PCR. Cells treated with JQ1 showed significantly less NF-κB target gene transcription than control cells (Fig. 5.3). Since JQ1 binds the acetyl-lysine binding pocket of Brd4, as shown by x-ray crystallography (Filippakopoulos et al., 2010), our findings suggest that interaction between acetylated p65 and Brd4 is required for *H. pylori*-induced NF-κB activation.

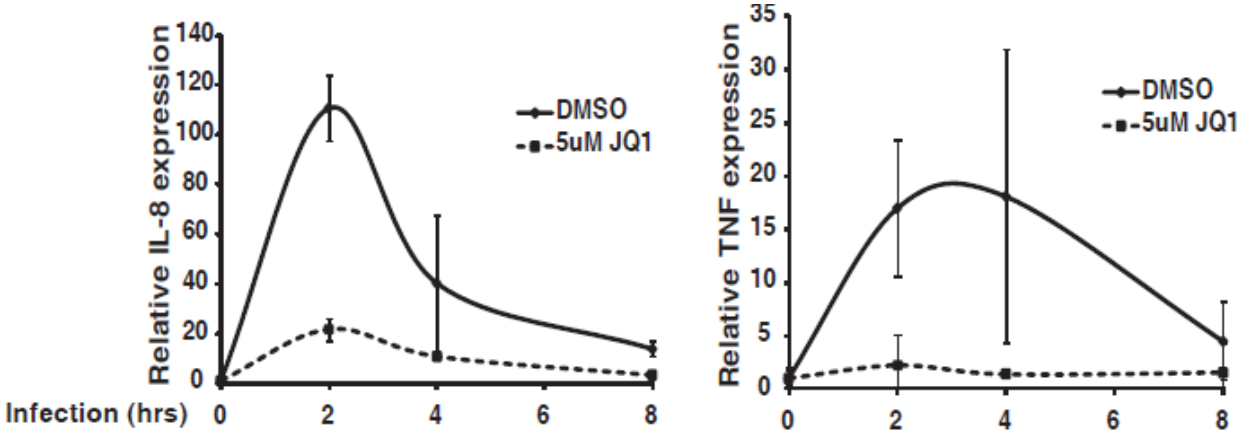

Figure 5.3. Small molecule JQ1 inhibits *H. pylori*-induced NF-κB activation. 5mM JQ1 or DMSO was added to AGS cells 30 min before *H. pylori* were added. Cells were infected with *H. pylori* for indicated times, and mRNA was harvested for RT-PCR.

5.4 Discussion

Post-translational modifications of NF-κB elaborate a "transcription factor code" much like the "histone code" proposed by Strahl and Allis in 2000 (Strahl and Allis, 2000). Regulation of the transcriptional activity of NF-κB can be achieved by the addition or removal of a number of molecules including phosphorylation, methylation, and acetylation. These modifications allow for co-factors of NF-κB to bind, dissociate, or promote further modifications which may increase or decrease transcription activity.

HDACs inhibited by TSA may be involved in deacetylation of many different sites within NF-κB or in deacetylation of the histones which are part of the chromatin near NF-κB target genes; therefore, it is possible that a site other than K310 may be acetylated in response *H. pylori* infection, or that inhibition of histone deacetylation may lead to the results I found. Since acetylation of histone tails typically results in activation of chromatin rather than suppression, the decreased transcriptional activity observed in the presence of TSA is more likely to be due to decreased acetylation of NF-κB. Seven lysines within NF-κB have been identified as acetylation sites. The negative effect of the HDAC inhibitor on *H. pylori*-induced NF-κB activity suggests that acetylation of sites which result in positive modulation of NF-κB activity are targets in *H. pylori* infection. Acetylation of K221 or K310, for example, enhance transcriptional activity of NF-κB (Chen et al., 2002). Besides these, acetylation of K314 and K315 may direct activation of specific target genes (Buerki et al., 2008; Rothgiesser et al., 2010).

Whether these modifications are specifically targeted by a *H. pylori* virulence factor is not known. While CagA appears to be necessary at least for phosphorylation (Fig. 2.6), it may be that it sets in motion a specific pathway of activations that includes certain pre-determined modifications required for full activation of the transcription factor, or for activation of a specific subset of NF-κB target genes. Another possibility is that CagA directly activates kinases, acetylases, or other enzymes required for post-translational activation of NF-κB. Since CagA singles out TAK1 in the upstream activation pathway of NF-κB, it is not impossible for it to also target downstream enzymes in this process. CagA is known to be a widely functioning protein, and has been seen in the nucleus of infected cells, the site of many post-translational modifications, by our lab.

Chronic activation of NF-κB, a regulator of inflammation and an inhibitor of apoptosis, is a hallmark of many cancers, and targeting NF-κB pathways to inhibit growth or induce apoptosis of these cancers may be a therapeutically useful goal. In this respect, small molecules such as JQ1, which are found to inhibit NF-κB by blocking co-factor binding to post-translational modifications of NF-κB, may be important in the future. Understanding the roles of post-translational modifications of transcription factors, and their regulation in normal and disease states, may be a critical piece of information for the treatment of cancers and other dysregulation-caused diseases.

CHAPTER 6

DISCUSSION

Helicobacter pylori infection is one of the leading factors for the development of gastric carcinoma. Infection with this common bacterium is often chronic, thriving for decades in the stomach of its host. Such a long incubation time allows for many changes to accumulate as a result of this interaction, and even small changes, such as subclinical inflammation, can cause great problems over a lifetime. Studying the pathways through which *H. pylori* virulence factors cause cellular or tissue changes, or activate signaling pathways such as the NF-κB, will be instrumental in better understanding host-pathogen interactions and also developing a better understanding of the ways in which inflammatory pathways are regulated.

In my studies, I found that CagA is required for activation of NF-κB during *H. pylori* infection. Infection of gastric epithelial cells with wild-type *H. pylori*, but not CagA-deficient *H. pylori*, enhanced NF-κB activity in luciferase assays and when NF-κB-dependent target gene mRNA was measured. Furthermore, various activating post-translational modifications of NF-κB are induced by *H. pylori* infection, including phosphorylation and acetylation. CagA likely activates NF-κB via upstream signaling through TRAF6- and Ubc13-mediated ubiquitination of TAK1, a kinase involved in phosphorylation and activation of the IKK complex. These studies have shed a great deal of light on the mechanisms involved in stimulation of the inflammatory pathways by *H. pylori*, and have opened new doors for better understanding of this host-pathogen interaction.

While our studies clearly demonstrate the importance of TAK1 ubiquitination and TAK1 activity in CagA-dependent *H. pylori*-mediated NF-κB activation, studies from others also suggest that other molecules might be targeted by CagA and involved in CagA-dependent NF-κB activation. Suzuki et al. reported that CagA interacted with the hepatocyte growth factor receptor Met, resulting in the activation of PI3K and Akt, which led to the activation of NF-κB and β-catenin (Suzuki et al., 2009). Since CagA binds Met, an intramembrane protein, and is also known to oligomerize, this binding and oligomerization may also lead to the recruitment of TRAF6. TRAF6 is recruited to the membrane by the dimerization of other membrane receptors, and it dimerizes in turn and becomes an active E3 ubiquitin ligase (Sun et al., 2004), which we found to be required for the ubiquitination of TAK1 (Fig. 4.7). Akt was also recently found to be ubiquitinated by TRAF6, which proved important for its phosphorylation and activation (Yang et al., 2009a). Although a role for Akt has been suggested in the activation of NF-κB by *H. pylori* (Suzuki et al., 2009; Takeshima et al., 2009), how Akt activates NF-κB is not clear. Akt might activate IKK directly or indirectly through TAK1, or Akt might activate NF-κB by inducing the phosphorylation of RelA. While TAK1 is activated in vitro by binding to unanchored ubiquitin (Xia et al., 2009), Akt might function as a kinase for the in vivo phosphorylation and activation of TAK1. It is also possible that Akt may act in conjunction with TAK1 to fully activate the IKK complex via phosphorylation of IKK1/2.

Although CagA enhances TRAF6-mediated Lys 63-linked ubiquitination of TAK1 (Fig. 3.5), it is not clear how this occurs. CagA fails to enhance the interaction between TRAF6 and TAK1 (Appendix Fig. A.1), excluding the possibility that CagA might function as a bridging factor to facilitate the TAK1–TRAF6 interaction. It is possible that CagA could target TRAF6 and enhance its E3 ligase activity or prevent TAK1 from interacting with deubiquitination

enzymes such as CYLD or A20, which remove ubiquitination and reduce activation of TAK1 (Reiley et al., 2007). Alternatively, CagA may prevent K48-linked ubiquitination by XIAP or Itch, preventing the turnover of the active enzyme, resulting in enhanced TAK1 activity (Ahmed et al., 2011; Kaur et al., 2005). It may also be possible that CagA interacts with the Ubc13 E2 enzyme complex, enhancing its activity or promoting its interaction with TRAF6 for the ubiquitination of TAK1. Further investigation into the mechanism of CagA-enhanced TAK1 K63-linked ubiquitination and activation would greatly enhance our understanding not only of *H. pylori* infection-induced activation of this NF-κB pathway, but would also help us understand the role and regulation of K63-linked ubiquitination more broadly.

Interestingly, TAK1 is involved in nearly every pathway that is activated by *H. pylori*, including the JNK, ERK and p38 MAPK pathways (Keates et al., 1999). We suggest that our CagA-dependent mechanism of activation for TAK1 in the NF-κB pathway may also contribute to the downstream activations of these other pathways, furthering our understanding of the role which CagA plays in host cells. Determining the precise way by which CagA stimulates the TRAF6-dependent ubiquitination and activation of TAK1 would clarify the *H. pylori*-induced inflammatory response, and would provide new information that could be used to combat development of the diseases linked to *H. pylori* infection.

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APPENDIX

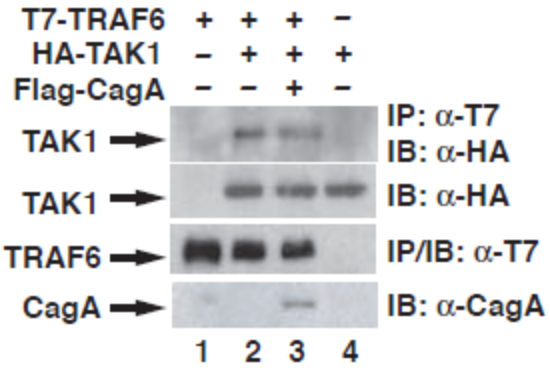

Figure A.1. CagA does not affect the interaction between TAK1 and TRAF6. HEK293T cells were transfected as indicated. T7-TRAF6 was immunoprecipitated, and immunoprecipitates were immunoblotted as indicated. Expression levels of each protein are shown in the lower three panels.

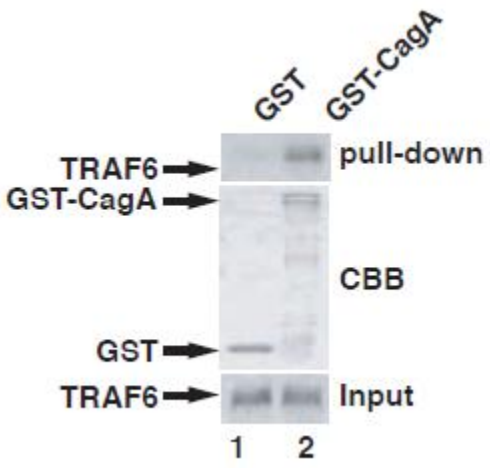

Figure A.2. CagA interacts with TRAF6 *in vitro*. GST and GST-CagA were used to pull-down *in vitro*-translated TRAF6. Pull-downs were separated by SDS-PAGE and TRAF6 was detected by autoradiography.

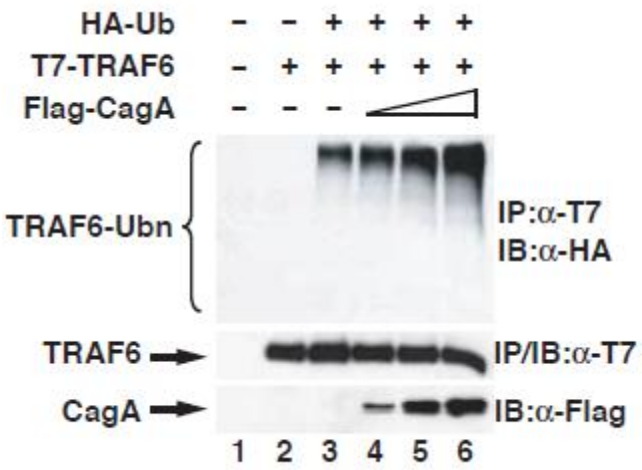

Figure A.3. CagA enhances the ubiquitination of TRAF6. HEK293T cells were transfected as indicated. T7-TRAF6 immunoprecipitates were immunoblotted for HA-ubiquitin and T7-TRAF6 as indicated. Levels of Flag-CagA are shown in the lower panel.